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MARY SWARTZ ROSE

1874 – 1941

MARY SWARTZ ROSE

OCTOBER 31, 1874 — FEBRUARY 1, 1941

An appreciation

The first of the former presidents of the Institute of Nutrition to pass from us was Professor Mendel, the second is one of his students whom he always regarded with an outstanding esteem, and who bore a distinguished part in the carrying on of his tradition and ideals, Doctor Mary Swartz Rose.

If in her case the doctoral rather than the professorial title comes first to mind, it is partly because she wore the former longer; and also because she exemplified so perfectly the definition of what the modern doctorate should mean—"a broadly educated person, sharpened to a point."

Only after a classical education in the liberal arts (Litt.B., Denison, 1901), did she turn her attention predominantly to science, and then with characteristic breadth of view she passed from the literary course of a classical college to the technical training of The Mechanics' Institute. Later, two years as student and assistant at Teachers College (B.S., 1906) with further study of food and nutrition in the Columbia department of chemistry, resulted in her definite, mature decision to make the science and teaching of nutrition her life work. To perfect her preparation for such a career she studied two years with Professor Mendel at Yale, receiving its Ph.D. degree in 1909.

Directly upon the completion of her work with Doctor Mendel she was appointed instructor in Teachers College, Columbia University, and became its first faculty member to devote full time to the teaching of nutrition and dietetics. The time was ripe for the development of this field, especially in the education of those who would teach nutrition and dietetics in the rapidly growing departments and schools of home economics

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in colleges and universities. Her success was immediate, and the rapid growth of her reputation was reflected in her successive promotions through the academic grades to the position of professor of nutrition; and in the numbers of exceptionally able people who came to study with her, and the eagerness with which they were sought for appointment to professorships in all parts of our continent and in many other parts of the world. She followed the tradition of Doctor Mendel's seminar method in her own; with its broad scientific scholarship and historical perspective in the arrangement of the readings, the discrimination with which they were assigned, and the skill with which the findings were finally summarized.

As a classroom and laboratory teacher of unified courses in nutrition and dietetics at the college and graduate levels she brought to bear her scientific and technical training and her life-long devotion to the art of teaching. Unquestionably a gifted teacher, she never permitted herself to rest upon the consciousness of her gift. She made her career an unceasing self-discipline, and always unreservedly and with keen alertness, she gave of her best in her daily work. The clarity and cogency of her exposition were the result of constant study and of systematic preparation. In addition she spoke with an infectious sincerity which inspired her students to be disciples and loyal coworkers.

Her knowledge and skill she gave also to the carrying of the new science of nutrition directly into public service; and on all fronts. Her public lectures and her writings—especially "Foundations of Nutrition" and "Feeding the Family" which so outstandingly combine interest, practicality, and scientific soundness—linked the findings of the nutrition laboratory with the daily lives of the people. Generously too, she joined in team-work for the further extension of nutritional knowledge and service through civic agencies and professional organizations—local, national, and international. She carried the message of what nutrition can mean for health and welfare into the public schools and the nursing and health centers of her community; she was long a member of the editorial board of The Journal of Nutrition, and was president of the Institute of Nutrition in 1937–1938; she served as a member of the

Council on Foods and Nutrition of the American Medical Association; and of the nutrition committee of the League of Nations. She was chosen by the international quarterly "Nutrition Abstracts and Reviews" to write a comprehensive account and interpretation of the college and university teaching of nutrition and dietetics in the United States. She served as deputy director of the bureau of conservation of the Food Administration in 1918–1919; and in 1940 was chosen one of a national group of five to serve as advisors on nutrition to the Council of National Defence, and consultants to the committee on food and nutrition of the National Research Council. These are but examples of her far-reaching service.

Her combination of breadth of view, depth of insight, and unsparing concentration of herself upon the effort in hand, is shown also in her record of research. Her versatility and wide reading are reflected in the range of her research topics; and their timeliness reflected the judgment of a critical and creative teacher as to what, at a given period, was most needed for the symmetrically strong development of our rapidly expanding science. Her critically constructive function in relation to the science of nutrition as a whole gave an underlying unity to her researches: in the utilization of foodstuffs; in the energy aspects of nutrition; in the nutritive values of foods as sources of protein, of calcium, and of vitamins; in the nutritional requirements for iron, with special reference to growth and development, and the nutritional availability of the iron of typical foods; in the more critically scientific determination of the nutritional characteristics of different groups of foods and the logical place of each in the diet. Planning her research as she did, with reference to the needs of our science as she saw it and without any anxiety as to individual fame, Mrs. Rose would doubtless deprecate any attempt to feature particular findings as her outstanding scientific contributions, and would say that her best discoveries are the students who have gone from her training into positions of leadership in the advancement of our science and of its functioning for human welfare.

H. C. S.

CEREALS AND RICKETS

XIII. PHYTIC ACID, YEAST NUCLEIC ACID, SOYBEAN PHOSPHATIDES AND INORGANIC SALTS AS SOURCES OF PHOSPHORUS FOR BONE CALCIFICATION [1]

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(Received for publication October 3, 1940)

Recent investigations in this laboratory (Krieger, Bunkfeldt and Steenbock, '40; Krieger and Steenbock, '40) revealed that in the absence of vitamin D the phosphorus of phytic acid was poorly available to the rat. In the presence of vitamin D its availability was markedly increased, although its efficiency never equalled that of inorganic phosphorus fed at the same level of intake. These findings are apparently important because approximately 40 to 70% of the phosphorus of cereal grains is present in the form of phytic acid (McCance and Widdowson, '35; Giri, '38).

In general the distribution of forms of phosphorus other than phytic acid in cereal grains has not been studied extensively. Attention, however, might be called to the following investigations. Heubner and Reeb ('08), Rising ('10) and Jebbink ('10) reported on the nucleic acid phosphorus present in grains and foodstuffs. Rengniez ('11) found that most of the phosphorus of flour was present as phytic acid and nucleic acid. Bernardini ('12) found both nucleic acid and phospholipid phosphorus present in small amounts in rice.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

Stellwaag (1890), Posternak ('03), Koch ('05), Suzuki, Yoshimura and Takaishi ('07) Heubner and Reeb ('08), Vageler ('09), Rising ('10), Vorbrodt ('10) and Alpers ('19) on the other hand turned their attention to the phospholipid content of cereals.

Apparently little is known of the comparative availability of these different forms of phosphorus. Accordingly in the following experiments the phosphorus of phytic acid from wheat bran was compared in its availability with that from yeast nucleic acid and soybean phosphatides using the rat as the experimental animal.

Unless indicated otherwise the basal ration employed was the low-phosphorus ration of Schneider and Steenbock ('39). This had a percentage composition of cerelose (glucose) 49, egg white 18, cooked starch 20, a rice polish concentrate [2] 4, phosphorus-free salts 4, and cottonseed oil 5. All additions to this ration were made at the expense of the cerelose. The phosphorus additions were based on the assumption that an optimal level of phosphorus intake was secured when 100 gm. of the ration contained 0.257 gm. of phosphorus (Krieger, Bunkfeldt and Steenbock, '40). The consumption of food was equalized for all rats at any one of the various levels of phosphorus fed. The rats used weighed from 50 to 60 gm. All experiments were run for a period of 4 weeks. The technique used was the same as that employed in previous investigations (Krieger and Steenbock, '40).

Nucleic acid phosphorus versus inorganic phosphorus. The yeast nucleic acid used in these experiments was a commercial product.[3] It contained 7.50% phosphorus as determined by the Fiske and Subbarow method ('25). It was apparently free from inorganic phosphorus because none was extracted by treatment with 2% HCl for 2 hours. The inorganic phosphorus additions were made in the form of a neutral mixture of the mono and dibasic potassium phosphates. Both the

[2] Vitab.

[3] Obtained from the Pfansteihl Chemical Company, Waukegan, Illinois.

nucleic acid phosphorus and inorganic phosphorus were fed at levels of 0.032, 0.064 and 0.128 gm. per 100 gm. of ration. In addition, both nucleic acid and inorganic phosphorus were fed at a level of 0.064 with the further addition of 45 U.S.P. units of vitamin D_2 to 100 gm. of ration.

It is evident (table 1) that the phosphorus of yeast nucleic acid was as readily available as inorganic phosphorus both in the presence and in the absence of vitamin D. The addition of vitamin D improved the utilization of both.

TABLE 1

The availability of nucleic acid phosphorus

GROUP	RATION COMPOSITION (ADDITIONS TO BASAL)	NUMBER OF RATS	FOOD CONSUMED	CHANGE IN WEIGHT	WEIGHT OF BONE	WEIGHT OF ASH	BONE ASH
			gm.	*gm.*	*gm.*	*gm.*	%
1	0.032 gm.[1] nucleic acid P	6	221	46	0.0998	0.0234	23.4
2	0.032 gm. inorganic P	6	208	37	0.0936	0.0213	22.8
3	0.064 gm. nucleic acid P	5	245	55	0.1153	0.0328	28.4
4	0.064 gm. inorganic P	4	241	53	0.1113	0.0315	28.3
5	0.064 gm. nucleic acid P plus vitamin D[2]	6	230	52	0.1245	0.0482	38.7
6	0.064 gm. inorganic P plus vitamin D	6	229	52	0.1263	0.0492	38.9
7	0.128 gm. nucleic acid P	6	259	70	0.1332	0.0518	38.9
8	0.128 gm. inorganic P	6	260	63	0.1345	0.0539	40.0

[1] Per 100 gm. ration.

[2] Forty-five U.S.P. units (viosterol) per 100 gm.

Further evidence of the equality of nucleic and inorganic phosphorus was obtained by inspection of the distal ends of the radii and ulnae of young rats after they had received these forms of phosphorus as a supplement to a rachitogenic diet. By way of preparation for the test, rats weighing 50 to 60 gm. were put on the low-phosphorus, rachitogenic diet of Schneider and Steenbock ('39). After 2 weeks on this diet when they were severely rachitic, they were given the supplements equivalent to 0.032 and 0.064 gm. of phosphorus in 100 gm. of ration. All the rats were given the same amount of ration.

After 5 days the amount of healing of the rachitic lesion was found to be the same for both supplements at their respective levels of intake.

Phospholipid phosphorus versus inorganic phosphorus. The phospholipid used for these experiments was a sample of re-precipitated crude soybean lecithin.[4] It was freed from most of the soybean oil carrier by extraction with acetone as follows: One hundred grams of the sample were triturated with 1000 cc. of acetone until a fine suspension was obtained. The phospholipid was then filtered off, re-extracted with 200 cc. of acetone and filtered again. The resulting waxy, light yellow product was dried in vacuo over calcium chloride in the dark. It was found to contain 2.63% of phosphorus. It was incorporated in the rations by dissolving it in ether and then evaporating the desired amount of the ether solution on the ration before a fan at room temperature. The experimental series was set up in the same manner as the preceding series with nucleic acid.

The results (table 2) show that in general phospholipid phosphorus was as available as inorganic phosphorus and that the addition of vitamin D improved the utilization of both to the same degree. However, at one level of intake, namely 0.064, inorganic phosphorus apparently produced slightly more bone ash than the phospholipid, namely 3.7%. It is possible that this difference was of no significance. However, it was deemed advisable to carry out an additional experiment. This was done by adding both sources of phosphorus to a basal ration low in both calcium and phosphorus. The calcium content of the ration was adjusted by the addition of calcium carbonate to the low phosphorus ration to give a calcium/phosphorus ratio of 1.5/1 and 10/1 respectively with the phosphorus at a level of 0.064 gm. per 100 gm. of ration.

The results of this experiment revealed slight differences in value of the phospholipid phosphorus at both calcium/phosphorus ratios. At the 1.5/1 ratio there were obtained

[4] Soya Lecithin XC—for this we are indebted to the American Lecithin Company, Inc., Elmhurst, Long Island, New York.

36.2 and 34.5% of bone ash respectively with phospholipid and inorganic phosphorus. At the 10/1 ratio the bone ashes were respectively 29.4 and 27.2%. When evaluated in relation to the slight differences in the other direction in the preceding experiments, the differences are believed to be without significance. Furthermore, inspection of the calcification of the radii and ulnae of the rachitic animals by the same technique as that employed before revealed the same amount of calcification in each.

TABLE 2

The availability of phospholipid phosphorus

GROUP	RATION COMPOSITION (ADDITIONS TO BASAL)	NUMBER OF RATS	FOOD CONSUMED	CHANGE IN WEIGHT	WEIGHT OF BONE	WEIGHT OF ASH	BONE ASH
			gm.	*gm.*	*gm.*	*gm.*	%
1	None	6	234	34.6	0.0899	0.0197	21.8
2	0.032 gm.[1] phospholipid P	6	252	53.0	0.1042	0.0251	24.0
3	0.032 gm. inorganic P[2]	6	252	56.6	0.1059	0.0279	26.6
4	0.064 gm. phospholipid P	6	250	63.9	0.1151	0.0346	30.2
5	0.064 gm. inorganic P	6	250	73.6	0.1221	0.0412	33.9
6	0.064 gm. phospholipid P plus vitamin D[3]	6	250	73.5	0.1446	0.0626	43.3
7	0.064 gm. inorganic P plus vitamin D	6	250	65.0	0.1435	0.0612	42.8
8	0.128 gm. phospholipid P	6	267	79.9	0.1447	0.0610	42.2
9	0.128 gm. inorganic P	6	267	89.5	0.1512	0.0629	41.6

[1] Per 100 gm. ration.

[2] As KH_2PO_4 instead of neutral phosphate mixture.

[3] Forty-five U.S.P. units (viosterol) per 100 gm.

Additional data on the equivalent value of phospholipid and inorganic phosphorus were obtained in an experiment involving both growth and reproduction. In this experiment one group of rats was fed phospholipid phosphorus and another the neutral mixture of inorganic phosphorus at such a level as to incorporate 0.11 gm. of phosphorus in each 100 gm. of the low phosphorus ration. Both rations were supplemented with 100 U.S.P. units of vitamin D (viosterol) per rat per week. Rats weighing from 50 to 60 gm. were put on these diets for a period of 20 weeks. They were then bred. The average weekly gain in weight for the rats on the phospho-

lipid phosphorus diet was 13.1 gm. for the males and 10.4 gm. for the females. For the rats on the inorganic phosphates the gains were 12.9 for the males and 9.3 gm. for the females. After mating, pregnancy was readily induced and normal young were born at term. One female on the phospholipid ration failed to rear her young, but three others maintained their litters for 9 weeks. The average weekly gain in weight for the young was 10 gm. on the phospholipid ration and 9.5 gm. on the inorganic phosphate ration.

TABLE 3

The availability of nucleic acid phosphorus, phospholipid phosphorus, inorganic phosphorus and phytic acid phosphorus

GROUP	RATION COMPOSITION (ADDITIONS TO BASAL)	NUMBER OF RATS	FOOD CONSUMED	CHANGE IN WEIGHT	WEIGHT OF BONE	WEIGHT OF ASH	BONE ASH
			gm.	*gm.*	*gm.*	*gm.*	%
1	0.064 gm.[1] nucleic acid P	6	196	44	0.1058	0.0349	33.0
2	Same plus vitamin D [2]	6	197	41	0.1283	0.0533	41.5
3	0.064 gm. phospholipid P	6	197	48	0.1003	0.0303	30.2
4	Same plus vitamin D	6	197	44	0.1182	0.0517	43.7
5	0.064 gm. inorganic P	6	197	44	0.1073	0.0349	32.5
6	Same plus vitamin D	6	197	41	0.1265	0.0523	41.3
7	0.064 gm. phytic acid P	6	194	42	0.0969	0.0229	23.6
8	Same plus vitamin D	6	197	44	0.1104	0.0411	37.2

[1] Per 100 gm. ration.

[2] Forty-five U.S.P. units (viosterol) per 100 gm.

Nucleic acid, phospholipid, phytic acid and inorganic phosphorus. The results in the preceding series, when evaluated in the relation to those obtained with phytic acid (Krieger, Bunkfeldt and Steenbock, '40; Krieger and Steenbock, '40), suggested a direct comparison of the aforementioned sources of phosphorus in one series. The nucleic acid and phospholipid preparations were the same as those used before. The phytic acid was prepared from phytin isolated from wheat bran according to the method of Boutwell ('17). Each preparation was added to the low-phosphorus basal ration in an amount equivalent to 0.064 gm. phosphorus per 100 gm. of ration. All rations were fed with and without vitamin D.

Table 3 reveals again that the phosphorus from yeast nucleic acid and soybean lecithin was the equal of inorganic phosphorus and that phytic acid was markedly inferior in this respect. Again the addition of vitamin D produced the greatest percentage improvement with phytic acid yet the resultant bone ash did not equal that obtained with the other compounds.

SUMMARY

The phosphorus of yeast nucleic acid and soybean phosphatides was found to be as readily available for calcification of bone as inorganic phosphorus. This stands in marked contrast with the availability of the phosphorus of phytic acid which is poorly available. By the addition of vitamin D the utilization of the phosphorus of phytic acid was increased in greater measure than that of the other forms of phosphorus but it was still less efficient for the production of good bone than the other sources.

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LIFE-TIME EXPERIMENTS UPON THE PROBLEM OF OPTIMAL CALCIUM INTAKE[1]

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(Received for publication October 10, 1940)

In experiments with rats, a mixture of five-sixths ground whole wheat and one-sixth dried whole milk with sodium chloride and distilled water (our diet A or laboratory diet 16) proves adequate, generation after generation, but is suboptimal in calcium and riboflavin content and in vitamin A value. In these respects it appears representative of a rather large proportion of human dietaries and we have therefore used this diet as a starting point in the experimental study of different dietary enrichments. (It is recognized, of course, that human dietaries may frequently be suboptimal in other respects also.)

EXPERIMENTAL

Our general experimental program includes enrichment of this original diet A or 16 in each of several factors (separately and in different combinations) up to the level of intake which induces its optimal response in nutritional well-being as measured by different criteria.

In previous papers it has been shown that when the calcium content is increased (without any other change in the diet than the addition of calcium salts) from the original 0.2%, to 0.35, 0.64, or 0.8% of calcium in the dry food mixture, there results in early life a relatively small increase in the rate of gain in

[1] We wish gratefully to acknowledge the assistance of grants from the Rockefeller Foundation and from the Carnegie Corporation of New York through the Carnegie Institution of Washington.

body weight and a relatively larger increase in the rate of gain in body calcium (Sherman and Campbell, '35, '37; Toepfer and Sherman, '36; Lanford and Sherman, '38; Sherman, Campbell and Lanford, '39); also that the 0.35% is distinctly superior to the 0.2% level throughout life (Sherman and Campbell, '35).

TABLE 1

Records of rats on diets of different calcium contents[1]

	MEANS ± THEIR PROBABLE ERRORS			
	On diet 168, 0.64% calcium		On diet 169, 0.8% calcium	
	Males	*Females*	*Males*	*Females*
Weight at 30 days, gm.	46 ± 0.4 (77)	45 ± 0.3 (105)	46 ± 0.4 (72)	45 ± 0.3 (104)
60 " "	116 ± 1 (77)	104 ± 1 (105)	119 ± 1 (72)	107 ± 1 (104)
90 " "	191 ± 2 (77)	159 ± 1 (105)	193 ± 2 (72)	160 ± 1 (104)
180 " "	303 ± 2 (77)	198 ± 1 (104)	305 ± 2 (72)	203 ± 1 (103)
365 " "	349 ± 3 (75)	231 ± 1 (99)	349 ± 3 (71)	237 ± 1 (101)
Age at birth of first young, days		111 ± 1 (102)		110 ± 1 (103)
Duration of reproductive life, days		311 ± 10 (105)		360 ± 10 (104)
Young born per female		38.6 ± 1.3 (105)		44.9 ± 1.35 (104)
Young reared per female		24.7 ± 0.9 (105)		27.0 ± 1.0 (104)
Avg. wt. of young at 28 days, gm.	40.2 ± 0.1 (2589)		40.1 ± 0.1 (2805)	
Length of life, days	734 ± 10 (77)	777 ± 13 (105)	729 ± 11 (71)	824 ± 11 (104)

[1] Number of cases given in parentheses under each mean.

The purpose of the present paper is to record the results of life-time and successive-generation experiments at the higher levels of 0.64 and 0.8% of calcium in the dry food; and to consider the question whether at these levels a definite "plateau of optimal nutritional response" (for such condi-

tions as these experiments represent) has now been located. The data here reported include experiments with 148 males and 209 females observed until natural death.

Table 1 compares the effects of the two diets here reported (diet 168 with 0.64%, and diet 169 with 0.8% calcium) upon body weight at different ages, duration of reproductive life, success of the females in bearing and rearing young, and length of life of each sex.

DISCUSSION

The weights at all ages from 1 month to 1 year, the ages of the females at the birth of their first young, the average weight of the offspring at 28 days of age, and the longevity of the males are substantially alike for these two diets. The females on the diet of higher calcium content, however, retained reproductive ability for a longer time, bore more young, reared more young, and lived longer. Any one of these differences taken alone and viewed in the light of its probable error may seem to be of doubtful statistical significance; but when all the observed differences fall to the credit of the same diet, it may be regarded as a matter for individual judgment whether or not the evidence shows a slight superiority in diet 169 over diet 168. As there were over 100 females on each of these diets, the effects of individual variation were presumably as well minimized as is practicable in long-time controlled experimentation.

Hence for such conditions as are here described, it appears that 0.64% of calcium in the dry food is either on or just at the beginning of the "plateau" of optimal intake for permanent nutritional well-being; and that 0.8% of calcium is certainly within this optimal region. How much above 0.8% this optimal zone extends, i.e., at what level a further increase of calcium intake would begin to be unfavorable in its effect upon permanent nutritional well-being, is a question not included in our problem.

CONCLUSION

In a dietary adequate in all respects but suboptimal in riboflavin content and vitamin A value a calcium intake of 0.64 to 0.8% of the dry food, or about three to four times that of minimal adequacy, gave best results in permanent nutritional well-being, as shown by full-life experiments in three generations of rats maintained under laboratory conditions.

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UNCOMPLICATED VITAMIN E DEFICIENCY IN THE RABBIT AND ITS RELATION TO THE TOXICITY OF COD LIVER OIL

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In 1931 Goettsch and Pappenheimer ('31) reported the production of extensive lesions of the voluntary muscles of rabbits and guinea pigs fed a natural food diet treated with ferric chloride, and supplemented with lard and cod liver oil (diet 13). Soon thereafter there appeared the first of a series of papers by the Cornell workers, later incorporated in a bulletin (Madsen, McCay and Maynard, '35), on the toxicity of cod liver oil for rabbits, guinea pigs, goats and sheep. These workers found that the addition of cod liver oil to synthetic and natural food diets produced muscle lesions, and though the dystrophic action of the synthetic diet (containing lard) was delayed when cod liver oil was omitted it was not entirely abolished. They concluded that both cod liver oil and some other factor of the synthetic diet were causative agents. Madsen ('36) noted the beneficial effect of cottonseed oil in dystrophy-producing diets. Davis, Maynard and McCay ('38) confirmed this observation, but found that cottonseed oil did not completely protect guinea pigs against dystrophy when cod liver oil concentrates were administered. They state that while it appears that a specific injurious factor is present in the oil, such an explanation must be considered tentative only.

We have shown (Mackenzie and McCollum, '39, '40) that muscular dystrophy in rabbits maintained on diet 13 plus

defatted wheat germ can be prevented or cured by alpha-tocopherol. Furthermore, we have been unable to confirm the report of Morgulis ('38) that a water-soluble factor is also required for maintenance of the integrity of the rabbit's skeletal muscles (Mackenzie, Levine and McCollum, '40). Morris ('39) found that rabbits rendered dystrophic on an alfalfa and grain diet through the oral administration of cod liver oil were cured by alpha-tocopherol. Shimotori, Emerson and Evans ('39, '40) were able to prevent dystrophy in guinea pigs fed a synthetic diet containing lard and cod liver oil through the administration of alpha-tocopherol. The California workers administered the cod liver oil and the alpha-tocopherol on alternate days. These results suggested that vitamin E, when properly administered, prevented the harmful action of cod liver oil on the voluntary muscles of the rabbit and guinea pig.

The next question in our investigations concerning the biological action of vitamin E was its mode of action in preventing the severe muscle lesions in the rabbit. Is it in itself an essential factor (readily inactivated by cod liver oil) in the metabolism of these animals, or does it protect them in some way from the toxic action of animal fats to which they are particularly sensitive? The delayed appearance of symptoms when cod liver oil was omitted from the synthetic diet (containing lard) of Madsen et al., the absence of dystrophy in goats and rabbits fed a vitamin E-deficient diet for more than a year by Thomas and Cannon (cited by Mattill, '38), and the months required to develop the neuro-muscular symptoms in rats reared on the most highly purified vitamin E-deficient diet yet used (Mackenzie, Mackenzie and McCollum, '40) suggested the latter explanation.

On the other hand, the observation on rats of Weber et al. ('39) that rancid fats destroy vitamin E when the two are mixed together in the diet, and that this destruction does not occur when rancid fats and vitamin E are fed separately 6 hours apart (the result of mixing in the gastrointestinal tract without previous contact in the diet was not studied) favored the first hypothesis. Mattill ('40) has reported that the vita-

min E content of a synthetic diet containing lard and cod liver oil gradually declines on standing, and that the loss of vitamin E may be delayed by the use of appropriate stabilizers against oxidative rancidity. He (Mattill, '38) has suggested that autoxidative rancidity of animal fats included in dystrophy-producing diets may be the principal cause of the disorder; vitamin E is antioxygenic in these fats and therefore it disappears in their presence even before rancidity becomes evident. Herbivorous animals have a large cecum where the food remains long enough for autoxidative changes to progress farther and more rapidly than in omnivorous animals. For a

TABLE 1

DIET	E 2	E 3
Casein (defatted)	150	150
Dextrin (cornstarch)	610	690
Lard (fresh)	80	
Yeast [1]	100	100
Salt mixture [2]	60	60
Carotene	0.05	0.1
Calciferol [3]	0.0004	0.0008

[1] Northwestern yeast foam tablet powder.
[2] Salt mixture No. 51 + Mn (Mackenzie, Mackenzie and McCollum, '39).
[3] Kindly supplied by Dr. C. E. Bills of Mead Johnson & Company.

discussion of the relationship between vitamin E, auto-oxidizing fats and antioxidants, the reader is referred to the review of Mattill ('38). So far as we know the destruction of vitamin E in the gastrointestinal tract or in the body by ingested cod liver oil has not been experimentally demonstrated.

The experiment reported in this paper was designed to test the effect on the rabbit of a vitamin E-deficient diet containing but traces of animal fat.

METHODS

The composition of the two basal diets used is given in table 1. Lard was included in diet E 2 as a source of the essential fatty acids and to determine the dystrophic effect of fresh lard. The casein was prepared by washing with acetic acid and

extracting in a Lloyd extractor with cold 70% and cold 95% alcohol for 24 hours each. It was the only source of animal fat in diet E 3 and according to the method of Shaw ('20), an adaptation of the Roese-Gottlieb procedure, contained but 0.25% of lipids.

The dextrin was ground in a Hobart burr mill with the burrs set at the number 5 position and was freed of the fine particles by sifting on a 20-mesh sieve. The carotene and calciferol were taken up in ethyl ether and evaporated on the casein and dextrin. The diets were mixed thoroughly and 125 cc. of tap water per kilogram was added to bind the components together. The rations were freshly prepared once or twice a week and were stored in the refrigerator. Roughage was not included in these diets because we wished to keep them as purified as possible. Previous experience with the components of both diets had shown that they were deficient in vitamin E.

Supplements. A solution of synthetic alpha-tocopherol [1] in ethyl laurate, 20 mg. per cubic centimeter, was employed as the source of vitamin E. The cod liver oil used was of a good medicinal grade. Both the tocopherol solution and the cod liver oil were kept in the refrigerator in glass stoppered flasks. The alpha-tocopherol was always administered by mouth from a 1 cc. tuberculin syringe equipped with a long blunt needle. The cod liver oil was either given in the same way or mixed in the diet daily.

Care of animals and diagnosis of dystrophy. The animals were housed in individual cages equipped with raised screen floors. They were weighed, given fresh diet, and their food consumption determined each day. At this time diet E 3 was again moistened with tap water. Contrary to our previous procedure the rabbits were allowed continuous access to the diets.

In these experiments we relied mainly on weight, food consumption and behavior for detecting the onset of dystrophy. Sections of voluntary muscles were examined microscopically in all cases at the end of the experiment. The muscle blocks

[1] We are indebted to Merck & Company, Inc., for the supply of alpha-tocopherol.

were fixed in Zenker-formol, imbedded in paraffin and stained with hematoxylin and eosin.

EXPERIMENTAL

Low fat and lard diets. Young rabbits weighing from 230 to 600 gm. were placed on diet 13 of Goettsch and Pappenheimer, from which the lard, cod liver oil and ferric chloride had been omitted. Beginning with the fourth day the purified diets were gradually added and by the eighth day the transfer to these diets was complete. At the end of this period, which is included in the experimental data, the controls were started on alpha-tocopherol at a level of 3 mg. a day, 6 days a week. Since the behavior of animals on diets E 2 and E 3 was similar, the results obtained on the two diets will be reported together.

The growth of rabbits on the purified diets supplemented with alpha-tocopherol averaged from 85 to 100 gm. a week. Although a loss in weight for 2 or 3 days frequently occurred, the weekly weights showed a consistent gain. Throughout the experiment some of the rabbits had watery feces intermittently for periods of several days. This seldom occurred in animals whose initial weight was less than 400 gm. Diarrhea was observed in only a few cases of severe dystrophy. The average daily food intake was 30 gm. during the first 3 weeks of the experiment and 35–43 gm. thereafter. While these diets were not optimal for growth in the rabbit, they proved quite suitable for our purpose. Lard was apparently not an essential component of the diet during the duration of the experiment.

The vitamin E-deficient animals grew as well as their controls for 3 weeks. They then either became very sick and lost weight rapidly or continued to grow, but at a reduced rate, throughout the remainder of the experiment. The average daily food intake of the surviving animals was 25 gm.

Gross symptoms of dystrophy (Mackenzie and McCollum, '40) appeared in all of the vitamin E-deficient rabbits in 2 to 4 weeks. Unlike the results obtained on diet 13 this was not always an indication of impending death. Except for the

muscular weakness, many of the animals appeared healthy 5 to 7 weeks later when they were sacrificed.

Microscopic examination of the thigh muscles and diaphragms from E-deficient animals killed at the twenty-third to seventieth day of the experiment invariably revealed severe degeneration. The muscles of the controls were normal. The

TABLE 2

The production of nutritional muscular dystrophy in young rabbits on a low fat diet (E 3), and on a low fat diet plus lard (E 2) and its prevention with alpha-tocopherol

RABBIT NO.	SEX	DIET	ALPHA-TOCOPHEROL[1]	INITIAL WEIGHT	MAXIMUM WEIGHT	TIME OF MAXIMUM WEIGHT	FINAL WEIGHT	DURATION OF EXPERIMENT	FIRST GROSS SYMPTOMS	MICROSCOPIC LESIONS	
										Thigh muscles[2]	Diaphragm
			mg.	*gm.*	*gm.*	*days*	*gm.*	*days*	*days*		
89	♀	E 3		550	930	70	930	70	30	++	++
91	♂	E 3	3	600	1510	100	1510	100		—	—
90	♂	E 3		590	950	70	950	70	21	+++++++++	++
92	♂	E 3	3	550	1560	100	1560	100		—	—
129	♂	E 3		360	580	32	520	37	17	++++	+++
128	♀	E 3	3	370	920	37	920	37		—	—
132	♂	E 3		320	680	37	680	37	29	++++	+++
133	♀	E 3	3	240	740	37	740	37		—	—
85	♀	E 2		410	780	21	620	23	22	++±	++++
86	♀	E 2	3	410	1320	55	1320	55		—	—
87	♀	E 2		340	640	26	510	29	16	++	++
88	♀	E 2	3	290	1800	105	1800	105		—	—
94	♀	E 2		540	1180	70	1180	70	28	+++++	+++
93	♀	E 2	3	580	1750	77	1700	100		—	—
95	♂	E 2		530	750	27	630	32	24	++	++
96	♂	E 2	3	490	1550	84	1500	100		—	—

[1] Alpha-tocopherol given 6 days a week. [2] Biceps femoris and vastus lateralis.

results obtained on a group of sixteen animals are summarized in table 2. It is apparent that the degree of muscle damage is not correlated with loss of weight or the duration of gross symptoms.

The lesions generally equaled in severity and extent those observed by us in a large series of rabbits rendered dystrophic

on diet 13. They were characterized by hyalinization and necrosis of the muscle fibers and by large accumulations of cellular elements, and were undistinguishable from those produced on diet 13. In several cases, however, the changes surpassed any that we had previously seen. The greater part of the muscle had been replaced by fibrous connective tissue containing an occasional intact muscle fiber with well-preserved cross striation. Scattered through the sections were hyalinized fibers and aggregates of cellular elements showing that degeneration was still in progress. The extensive disappearance of muscle fibers, with their replacement by connective tissue, was undoubtedly a consequence of the prolonged life of severely dystrophic animals on the purified diet as contrasted with diet 13. It was certainly not due to prolonged chronic dystrophy (Mackenzie, Levine and McCollum, '40).

The effect of cod liver oil on the low fat diet supplemented with alpha-tocopherol. Young rabbits were fed diet E 3 plus 3 mg. of alpha-tocopherol daily by mouth. Either 2 cc. of cod liver oil was mixed in the diet daily or 1 cc. was administered orally soon after the daily alpha-tocopherol supplement. The results were the same in both cases.

During the 5 weeks that the animals were kept on this regimen growth and food consumption paralleled that observed on the low fat vitamin E-deficient diet. Physical symptoms of dystrophy appeared at the fourth week. The daily creatine excretion on the thirty-fourth and thirty-fifth days was from 70 to 80 mg. per kilogram of body weight. Microscopic examination of voluntary muscles taken when the animals were killed at 5 weeks revealed lesions at least as severe as those produced on diet E 3 in the same length of time. These lesions, scored as 4 or 5 plus, were similar to those produced on diet E 3 without alpha-tocopherol or cod liver oil. It should be noted here that the consumption of 2.9 gm. of lard daily (diet E 2) for 14 weeks produced no lesions in rabbits receiving alpha-tocopherol at the 3 mg. level.

DISCUSSION

Young rabbits fed a vitamin E deficient diet containing 0.05% of animal fat develop severe lesions of the voluntary muscles. These lesions are prevented by synthetic alpha-tocopherol. It may be concluded, therefore, that the ingestion of animal fats (cod liver oil in particular) is not a prerequisite for the production of nutritional muscular dystrophy in rabbits, and that vitamin E is essential for the normal structure of their voluntary muscles.

The preventive action of alpha-tocopherol, administered orally, was rendered ineffective by the oral administration of cod liver oil a few minutes later or its addition to the basal diet. Muscle lesions were produced resembling those observed on the low fat, vitamin E-deficient diet. Fresh lard added to the diet did not possess this dystrophic action.

The dystrophic action of cod liver oil fed separately from the vitamin E may be explained in several ways: (1) The destruction of alpha-tocopherol by cod liver oil following the ingestion of these two substances. (2) A toxic action of cod liver oil, unrelated to vitamin E, but producing similar morphological changes in the voluntary muscles. (3) A destructive action of cod liver oil on the muscles that is prevented by vitamin E either by virtue of its vitamin activity, or its activity as an antioxidant (Olcott and Emerson, '37) if indeed these two functions are independent. In this case the vitamin requirement should parallel the cod liver oil intake.

The observation of Shimotori et al. ('40) that guinea pigs on a purified diet plus cod liver oil are protected against dystrophy by alpha-tocopherol (the alpha-tocopherol and cod liver oil being administered on alternate days), and the experiments reported in this paper in which the action of alpha-tocopherol was rendered ineffective by cod liver oil when no precautions were taken to prevent the mixing of the vitamin and the oil in the gastrointestinal tract make the first explanation the most likely. If this should prove to be the case it would probably also account for the action of cod liver oil on the voluntary muscles of other animals. However, additional work is

needed for the establishment of this hypothesis as the correct one. This is particularly so in view of the report of Davis, Maynard and McCay ('38) on the dystrophic action of cod liver oil concentrates. It must be borne in mind that the possible explanations offered above do not necessarily exclude one another.

SUMMARY

1. Vitamin E deficiency in rabbits maintained on a purified low fat diet results in the rapid development of severe lesions of the voluntary muscles. These lesions are prevented by synthetic alpha-tocopherol.

2. The preventive action of alpha-tocopherol is rendered ineffective when the vitamin and cod liver oil are administered orally within a few minutes of each other at the levels employed, or when the oil is added to the basal diet.

The histological preparations used in this experiment were made by Miss Miriam Reed.

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RELATION OF PARATHYROID FUNCTION AND DIET TO THE MINERAL COMPOSITION OF THE BONES IN RATS AT THE CONCLUSION OF PREGNANCY [1]

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ONE FIGURE

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The complications developing toward the end of pregnancy in parathyroid-deficient rats appear to be related to the marked depression of the serum calcium. A diet may be adequate for the normal pregnant rat and fairly so for the parathyroidectomized non-pregnant rat, yet fail to maintain the calcium concentration of the blood of the parathyroid-deficient pregnant rat at anywhere near the normal level. From this it may be deduced that regulation of the blood calcium in the pregnant organism depends, even more than in the non-pregnant animal, on its ability to mobilize calcium from its reserves or from a high calcium intake. In order to show how parathyroid deficiency affects the calcium and phosphorus economy of pregnant animals, the mineral composition of the bones of parathyroidectomized rats following one or more pregnancies was compared with that in unoperated control rats.

It is common knowledge that demineralization of bone occurs during pregnancy, especially if the mineral composition of the diet falls short of the requirements. It is also recognized that

[1] This work was aided by generous grants from the Mead Johnson Company, Evansville, Indiana, and approved by the Council for Pediatric Research of the American Academy of Pediatrics.

such changes as may accompany a single pregnancy are augmented by repeated pregnancies, and are, therefore, more readily demonstrable. This is illustrated by the data in table 1 which permit comparisons between parathyroidectomized rats and their normal pregnant controls: (1) on diet no. 7 (Ca 0.49%, P 0.49%), two pregnancies; (2) on diet no. 7, three pregnancies; (3) on diet no. 27 (Ca 0.122%, P 0.245%), three pregnancies. The experimental procedure and diets are described in a previous paper ('41).

The data in table 1 bring out several significant points which are more amply confirmed by the data represented in figure 1 and by those in table 2. The tabulated data show that in our experiments the parathyroid-deficient animals differed from the corresponding controls in two important respects. The ash content of the bones (humeri and femurs, dried and defatted) tended to be higher in the parathyroid-deficient animals than in the controls. Even more conspicuous (tables 1 and 2; fig. 1) were the heavier weights, both absolute and relative to the pregestational body weight, of the bones (two humeri and two femurs) in the parathyroid-deficient rats. At this point it is necessary to explain that the last pregestational weight was taken as a more reliable basis for calculation than the weight at any stage of pregnancy or that following parturition. This convention eliminated errors due to different rates of gain in weight during pregnancy in normal and parathyroidectomized rats, variable loss of weight toward the end of gestation in parathyroidectomized and rarely in normal rats because of loss of appetite during the last 2 or 3 days, differences in size and weight of litters, and other factors.

From the data in table 1, it is seen that the coefficient, bone weight/body weight $\times$ 1000, and the ash content of the bones were invariably higher for the parathyroprevic rats than for the corresponding controls.

The data for rats 324 and 354 are included for the purpose of recording the lowest bone weight/body weight coefficients encountered in the present series of experiments. As may be anticipated, these occurred in unoperated control rats maintained for long periods on rations deficient in calcium. One

had been fed diet no. 27 (Ca 0.122%, P 0.245%) for a period of more than 7 months, during which time it gave birth to four litters; the other had been fed diet no. 26 (Ca 0.017%, P 0.245%) for a shorter period (104 days), during which time it gave birth to three litters. The ash content and the per cent of calcium of the bones were significantly lower than in the unoperated pregnant rats fed diet no. 7.

The curves in figure 1 are based on the results obtained in normal and parathyroid-deficient pregnant rats maintained

TABLE 1

Influence of repeated pregnancies on composition of bones in parathyroid-deficient (P-D) rats and their normal controls (C)

Rat no.	529 C	458 P-D	304 C	447 P-D	364 C	78 P-D	354 C	324 C
Diet no.	7	7	7	7	27	27	26	27
Duration of diet, months	2	3⅔	4½	8½	4	5	3½	7½
Number of pregnancies	2	2	3	3	3	3	3	4
Per cent of ash[1]	64.75	66.56	66.00	69.09	62.10	66.60	54.75	58.03
Calcium, per cent of ash[1]	36.80	36.77	36.18	36.55	36.32	38.66	34.32	35.84
Phosphorus, per cent of ash[1]	18.46	18.68	17.65	16.23	17.96	17.88	17.50	17.12
Calcium-phosphorus ratio	1.99	1.97	2.05	2.25	2.02	2.16	1.96	2.09
Weight of bones[1] (2 humeri, 2 femurs) in gm.	1.094	1.302	1.154	1.387	1.045	1.345	0.728	0.867
Last pregestational body weight in gm.	198	198	240	221	196	217	205	224
$\frac{\text{Bone weight}}{\text{Rat weight}} \times 1000$	5.53	6.57	4.81	6.27	5.33	6.20	3.55	3.87

[1] Footnote to table 2 applies also to the data in this table.

on diet no. 7, the calcium and phosphorus content of which is such as to assure maximum reproductive success in normal rats (Cox and Imboden, '36; Bodansky and Duff, '41). The lower curves represent the observations in animals that had passed through one successful reproductive cycle, while the upper curves represent observations in animals that had gone through two such cycles. Admitting that the numbers of observations in each of these subgroups were small and that the bone weight/body weight coefficients showed a wide range of

individual variation, the results reveal, nevertheless, the important effect of intact parathyroid function on the mineral reserves of bone during pregnancy. Thus, at the conclusion of one pregnancy the mode for the bone weight/body weight coefficients of fifteen rats with intact parathyroids was approximately 5.3, while that for thirty-nine parathyroidectomized rats was approximately 6.0. A second pregnancy caused an even further reduction in the mean and modal values for the bone weight/body weight coefficients of the normal rats.

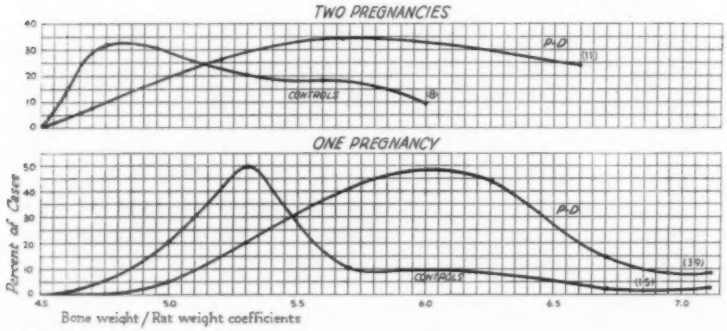

Fig. 1 Curves showing the effect of presence or absence of parathyroids on the weight of the bones (femura and humeri; dried, alcohol and ether extracted) in rats at the conclusion of one and two pregnancies. Abscissas represent the ratios: bone weight/pregestational body weight × 1000. Ordinates represent the per cent of rats in each category. Curves for the parathyroid-deficient rats are designated by the letters P–D. The figures in parentheses denote the number of rats in each group.

In table 2 are summarized the data obtained on the various diets. It will be seen that, excepting diet no. 16, the bone weight/body weight ratios were invariably lower in the controls than in the parathyroid-deficient rats. The long bones of the latter, on the different diets, averaged 10 to 20% heavier than those of the controls. There was, moreover, a higher ash content in the bones of the parathyroid-deficient animals. In contrast to these findings, the average bone weight coefficient and the average per cent of ash were of approximately the same magnitude for both the operated and unoperated rats fed diet no. 16 (Ca 1.225%, P 0.245%, Ca/P

TABLE 2

Influence of pregnancy on composition of bones in parathyroid-deficient (P-D) rats and their normal controls (C)

DIET NO.		NUMBER OF ANALYSES	PER CENT ASH	COMPOSITION OF ASH [1]		AVERAGE Ca/P RATIOS	BONE WEIGHT / RAT WEIGHT × 1000
				Per cent Ca	Per cent P		
7	C	28	65.25 ± 2.21	36.82 ± 1.27	18.32 ± 0.67	2.01	5.426 ± 0.669
	P-D	45	66.62 ± 2.23	36.31 ± 1.54	18.23 ± 0.81	1.99	6.023 ± 0.573
8	C	4	65.12 ± 2.62	36.56 ± 0.76	18.16 ± 1.22	2.01	4.712 ± 0.339
	P-D	2	67.22 ± 0.04	36.57 ± 0.61	17.94 ± 0.46	2.04	5.503 ± 0.038
10	C	5	65.20 ± 1.83	37.04 ± 1.82	17.92 ± 1.02	2.07	5.052 ± 0.558
	P-D	1	67.50	36.78	17.46	2.11	6.305
12	C	6	66.12 ± 2.21	36.86 ± 0.93	18.01 ± 1.08	2.05	5.146 ± 0.489
	P-D	7	66.93 ± 2.60	37.09 ± 0.94	18.21 ± 0.92	2.04	5.833 ± 0.356
16	C	7	63.99 ± 1.91	37.11 ± 0.45	18.58 ± 0.83	2.00	5.360 ± 0.232
	P-D	8	64.25 ± 3.34	36.95 ± 0.31	18.50 ± 0.99	2.00	5.264 ± 0.394
26	C	15	63.53 ± 3.50	37.60 ± 0.72	18.26 ± 1.24	2.06	4.738 ± 0.522
	P-D	9	65.69 ± 2.15	36.52 ± 0.48	18.88 ± 0.48	1.93	6.032 ± 0.362
27	C	11	63.41 ± 1.88	37.24 ± 1.41	18.02 ± 0.58	2.07	5.297 ± 0.347
	P-D	13	64.98 ± 2.47	37.25 ± 1.55	18.17 ± 0.31	2.05	6.229 ± 0.362
19	C	5	66.55 ± 1.57	36.75 ± 0.43	18.75 ± 0.41	1.96	5.609 ± 0.411
	P-D	4	68.86 ± 1.35	36.04 ± 0.39	18.72 ± 0.37	1.93	6 312 ± 0.402

[1] Calcium was determined by titration of the oxalate with permanganate, following the technique of A. T. Shohl and F. G. Pedley (1922, J. Biol. Chem., vol. 50, p. 537). Phosphorus was determined by adapting the method of A. T. Shohl and H. B. Brown, as described by Van Slyke and Peters in Quantitative Clinical Chemistry, vol. 2, p. 869.

Preliminary to analysis, the bones were freed from as much tissue as possible, dried at 105°C. to constant weight, scraped carefully, without loss of bone substance, broken into several pieces and extracted for 18 hours with boiling alcohol and then for 18 hours with boiling ether. The bone weights, as well as the analytical data, represent the alcohol- and ether-extracted, dried bones.

ratio 5.0). The explanation presumably lies in the fact that this diet is rachitogenic and that its effects in promoting mobilization of bone salts and in retarding bone growth more than offset the effects of parathyroid deficiency. On diet no. 16 (Bodansky and Duff, '41), the pregnant rats did not exhibit the usual symptoms of parathyroid deficiency, the serum calcium being maintained at a high level throughout pregnancy.

DISCUSSION OF RESULTS

In interpreting the bone changes, consideration must be given to the fact that the rats used in our experiments had not attained full growth. Hammett ('23, '24) has shown that anatomical and chemical differentiation of bone is significantly altered in the growing female rat in the absence of parathyroid secretion. He studied particularly the changes produced during the 50-day interval between 100 and 150 days of age. In females, as in males, lack of parathyroid function caused moderate retardation of absolute growth in weight and length of the humerus and femur. No distortion of anatomical or chemical differentiation of these bones was apparent in the male rats, but in the females, the long bones were not only retarded in growth, but contained a lower per cent of ash, a lower per cent of calcium, and somewhat higher percentages of phosphorus and magnesium than in normal animals.

These changes, characteristic of the parathyroid-deficient, growing rat, were more than offset in our experiments by the effects of pregnancy. The mechanisms which come into play during pregnancy tend to produce in rats with intact parathyroid function demineralization and retarded bone growth to such a degree that the bones (femurs and humeri) weigh less and have a lower mineral content than the corresponding bones of the parathyroid-deficient pregnant rats.

The significance of these findings is apparent. In response to pregnancy the normal rat draws, to a considerable degree, on the calcium and phosphorus reserves of the bones and perhaps on other sources, while in rats without parathyroids, the usual mechanism for the mobilization of bone salts is abolished. The failure of this mechanism is clearly brought out by the

close similarity of the bone weight coefficients for the parathyroidectomized pregnant rats fed rations as diverse in calcium content as 0.49% (diet no. 7), 0.122% (diet no. 27), and 0.017% (diet no. 26). On the other hand, in rats with intact parathyroids significant differences occurred on these diets. As shown in table 2, the average bone weight coefficient was somewhat higher for the control rats fed diet no. 7 than for those fed diet no. 27 and distinctly higher than for those fed diet no. 26.

From these considerations it does not follow that the contrasting bone changes in the parathyroidectomized and control rats were necessarily due to abnormal excretion of calcium in the latter group. Moderate degrees of decalcification may in fact accompany positive calcium and phosphorus balances, indicating a possible relationship to the redistribution of minerals incidental to continued body growth. The normal pregnant rat frequently shows a considerable absolute gain in weight during pregnancy, whereas the parathyroid-deficient rat, despite equivalent food consumption, usually shows little or no change.

The average calcium-phosphorus ratios of the ash fell within a narrow range of variation, were apparently unaffected by the composition of the diet, and did not appear to be significantly different in the control rats than in the parathyroid-deficient rats (table 2). However, occasionally, high values were encountered in individual cases (for example, rats 78 and 447, table 1).

In planning our experiments, the main objective was to determine the effects of parathyroid deficiency in the pregnant rat. Unfortunately, we did not anticipate the possible value of data obtained in virgin rats fed the same diets, although analyses were made of the bones of rats which failed to become pregnant. On the basis of the data thus obtained, certain statements may be made tentatively. In rats with intact parathyroids the bone weight coefficients tended to be somewhat higher for the non-pregnant rats than for the pregnant rats, the greatest discrepancy occurring when the calcium intake was very deficient (diet no. 26). This relation seemed

to be reversed in the parathyroidectomized rats. Thus, the average coefficient for eight sterile rats on diet no. 7 was 5.77, compared to 6.023 for the pregnant group (table 2). The average for nine sterile rats on diet no. 26 was 5.71, compared to 6.032 for the pregnant group. The full significance of these differences is obscure, though they may be related partly to differences in food consumption and rates of growth.

SUMMARY

Failure to maintain the serum calcium at a normal level in the pregnant, parathyroidectomized rat suggests a deficiency in the mobilization of calcium from bone. This deficiency in mobilization of the bone salts is also demonstrated by the finding that in general the long bones obtained at the conclusion of a pregnancy weighed more, in relation to the pregestational body weight, in the parathyroid-deficient rats than in the controls. The per cent of ash of the bones was likewise higher in the parathyroid-deficient rats than in the controls, while the calcium-phosphorus ratios were, on the average, nearly identical.

These relations held for various levels of calcium and phosphorus intake, the exception being diet no. 16 of Cox and Imboden. On this diet, which is rachitogenic because of its high calcium content and high calcium-phosphorus ratio, the disparity in the bone weight coefficients between normal and parathyroidectomized pregnant rats was abolished.

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A DEFICIENCY DISEASE OF FOXES PRODUCED BY FEEDING FISH

B_1 AVITAMINOSIS ANALOGOUS TO WERNICKE'S DISEASE OF MAN[1]

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Chastek paralysis, a dietary disease in which the principal clinical and pathologic characteristics are referable to the central nervous system, has become a major disease problem of the fox-raising industry. Clinically, the syndrome is characterized by anorexia, weakness, progressive ataxia and spastic paraplegia, with death occurring within 48 to 72 hours after the onset of neurologic symptoms. Hyperesthesia is common and the foxes seem to be in great pain, as they moan continuously in the advanced stages of the disease. At autopsy the liver usually shows severe degeneration and in the brain diagnostic vascular lesions identical with those of Wernicke's disease of man are found bilaterally and are located principally in the paraventricular grey matter.

A brief description of this disease and of its relation to Wernicke's hemorrhagic polioencephalitis has been published (Green and Evans, '40) and a report of pathologic studies is now being prepared for publication. A general description of several outbreaks of this disease and a consideration of the nutritional factors involved are the subjects of this paper.

[1] This study was carried on with the aid of the Minnesota Department of Conservation.

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Rations employed on fox farms vary considerably but are usually made up of a thoroughly ground mixture of either horse or rabbit meat, ground cereal, and a variable proportion of vegetables such as carrots and tomatoes. Fish is not generally used, but an occasional fox farmer has added it to the ration as an economical substitute for part of the meat component.

Careful study of the dietary records on ranches where Chastek paralysis occurred convinced us several years ago that the cause of this disease was the feeding of fresh fish (usually frozen) as 10% or more or the ration. From pathologic studies, as well as from dietary records on ranches where outbreaks were observed, we were led to the further conclusion that the foxes were actually dying of a vitamin-B_1 deficiency. Although these two latter statements appeared to be so unrelated as to be almost inconsistent, data supporting each were sufficiently strong to warrant experimental study. A recently completed experiment confirms both conclusions in their entirety.

In 1932 and in 1936, groups of foxes totaling about 300 animals were fed diets containing fish. Chastek paralysis was produced in some groups and not in others. This year we have learned that the disease may be consistently produced by a ration containing fish if the foxes are placed in pens with elevated, screened floors, so that their feces fall to the ground and no grass is available to them.

In an experiment begun on July 12, 1940, and terminated on August 25, forty-six foxes were used. All animals were kept in pens with elevated, screen floors throughout the experiment. A diet of the sort which is known to be entirely satisfactory for the raising of foxes was made up and to this was added frozen carp in the amount of 20% of the ration.

The basic diet in this experiment had the following percentage composition: cereal 15, bread 10, carrots 9, horse meat 45, carp 20, and cod liver oil 0.5. The cereal was a mixture of ground wheat, corn, oats and rice. The meat was all horse meat from frozen supplies except on 2 days when recently slaughtered animals were used. The carp was used whole—

heads, scales and viscera being included. All ingredients of the diet were ground together to a mixture approximating the consistency of hamburger.

The foxes used had been on the regular ranch diet prior to the time the experiment was begun. On July 12 these animals were divided into three groups and given diets as follows: One group of eighteen foxes received the basal diet plus 20 mg. of nicotinic acid for each fox daily. In a second group of sixteen, each animal was fed the basal diet plus 25 mg. of thiamine hydrochloride daily. Nothing was added to the basal diet for a third group of twelve foxes.

All foxes receiving the basal diet alone and all those being fed supplements of nicotinic acid went off feed on July 24, 12 days after the experiment was begun and remained off feed until it was ended. Only those foxes receiving the basal diet plus thiamine hydrochloride maintained normal appetites and remained symptom-free and in good health throughout the experiment.

Fatalities began to occur in the control group receiving no supplement of vitamins on August 1, 20 days after the experiment began. Seven of the twelve foxes in this group were dead of typical Chastek paralysis by August 15, a mortality of more than 50%.

In the group fed the basal diet plus nicotinic acid, two foxes died on July 30, two more died on August 1, and eleven of the eighteen foxes had died of Chastek paralysis by August 18.

Foxes in the third group were fed exclusively on the basal ration plus thiamine until August 25 and remained entirely normal throughout the experiment.

Results of this experiment show firstly that Chastek paralysis may be produced at will by feeding a diet containing 20% fresh carp. In no case have we observed an outbreak of Chastek paralysis on a ranch where fish or fish products constituted less than 10% of the total ration and, since most fox ranchers do not feed fish to their animals, this fact alone provides strong evidence that Chastek paralysis is caused by the

feeding of fish. All available experimental evidence further supports this conclusion.

Because none of the sixteen foxes receiving the basal diet plus the supplement of thiamine hydrochloride showed any evidence of illness and inasmuch as Chastek paralysis was allowed to progress until it had caused a mortality of more than 50% in each of the other two groups, it is clear that thiamine hydrochloride protects foxes from the disease. Before discussing the possible mechanism by which fresh fish could bring about a deficiency of vitamin B_1, we shall present a description of some of the ranch outbreaks observed. It should be noted in reading these brief histories that the disease followed, in all cases, the feeding of fish.

Ranch outbreaks of Chastek paralysis

Our first observations of Chastek paralysis were made in the spring of 1932 on a ranch owned by Mr. J. S. Chastek near Glencoe, Minnesota. Fish was first used in the ration on this ranch on January 21, 1932, when frozen carp was added to the feed in an unknown proportion. The foxes were well until February 16 when they were reported "off feed". The first death occurred 1 week later, on February 23. By March 17 a total of sixty-eight foxes, or 34% of the herd of 208, had died and many of the remaining animals were ill. Because the role of fish as a cause of the disease was unknown at that time, the diet was essentially unchanged until March 17 when fish was removed from the ration and increased amounts of liver were added. After that date only two foxes died. Foxes too ill to eat after March 17 were treated by intraperitoneal injections of saline and by tube-feeding water and milk.

During the winter of 1936 two fox farms near Logan, Utah, experienced outbreaks of Chastek paralysis. On both, frozen carp was used as part of the ration beginning on January 10. On one, the L. ranch, 30% of the diet was carp; and on the other, the R. ranch, 10% was carp. About 1 month later, symptoms of Chastek paralysis developed on both ranches and one death occurred on the R. ranch before February 13, the date

fish was eliminated from the rations of both ranches. As a result of a prompt dietary change, the mortality on these two ranches was relatively low. Of the 544 foxes on the L. ranch, 1.8% died; and of the sixty-nine foxes on the R. ranch, 5.8% died. The diets that induced Chastek paralysis on these ranches are listed in table 1.

In March and April, 1939, an outbreak of Chastek paralysis occurred on a silver fox farm near Grand Forks, North Dakota. A total of 236 foxes (140 females and 96 males) was present on the ranch before the outbreak. Twenty-eight (twenty-two males and six females) died. A complete dietary schedule from

TABLE 1

Diets which caused Chastek paralysis on two fox ranches in Utah, 1936

	L. FOX FARM	R. FOX FARM
	%	%
Fish	30	10
Red meat (horse and beef)	30	40
Tripe		10
Prepared cereal	15	25
Laurel wheat	15	
Carrots	10	15

3 months prior to the outbreak until the disease had entirely disappeared, is shown in table 2.

Feeding Lake Superior herring on 4 days a week and no fish on 3 days a week for nearly 2 months did not in any way harm the foxes or predispose them to Chastek paralysis. The disease first appeared about 3½ weeks after "mullets or suckers from Lake Manitoba" were substituted for the herring and the ration was changed to include fish 7 days a week instead of 4 days a week.

Although the foxes ate poorly after March 12, no deaths occurred until March 27 when one male died. The next day a second male died. A more varied diet, which did not include fish, was adopted on March 28 and was maintained until the disease had completely disappeared. After this change in diet, there was a rapid improvement so that the foxes were soon

eating well. However, the disease had apparently advanced so far in some animals that the change in diet did not cure them. As a result, there were twenty-six deaths from March 30 through April 10. The disease in this instance may be attributed solely to the mullets or suckers fed as 15% of the diet 7 days a week beginning February 15.

TABLE 2

Rations fed on a silver fox farm near Grand Forks, North Dakota, 1938–39

	DEC. 20 TO FEB. 14[1]	FEB. 15 TO MAR. 17	MAR. 18 TO MAR. 24	MAR. 25 TO MAR. 27	MAR. 28 UNTIL AFTER DISEASE DISAPPEARED
Meat	70%	70%	70%	100%	70% [2]
Beef melts		15 lb.			
Horse meat	90 lb.	80 lb.	90 lb.	100%	
Fish [3]	30 lb.	40 lb.	40 lb.		
Horse entrails	15 lb.	20 lb.	20 lb.		
Horse liver		10 lb.	10 lb.		
Beef tripe	10 lb.	15 lb.	15 lb.		
Purina meal	20%	20%	20%		20%
Ground green bone	5%	5%	5%		5%
Lettuce and carrots	5%	5%	5%		5%
Supplementary ingredients					
Milk	3 gal.	5 gal.	2 gal.		3½ gal.
Dry brewer's yeast	3 lb.	3 lb.	3 lb.		3 lb.
Cod liver oil	1 pt.	1 qt.	½ pt.		1 pt.
Dried figs		4 lb.	3 lb.		2 lb.
Fresh eggs		5 doz.	5 doz.		5 doz.

[1] Mixed feed was fed 4 days a week and pieces of horse meat, 3 days a week.
[2] The 70% meat includes all kinds named except fish and horse entrails.
[3] Lake Superior herring was fed until February 14; mullets or suckers from Lake Manitoba were fed from February 15 through March 24.

Chastek paralysis developed during January, 1940, on a ranch near Coon Rapids, Minnesota, where the herd consisted of seventy foxes, or thirty-five pairs. The diet on this ranch was 20 or 30% commercial feed and 70 or 80% rabbit carcasses. On January 15, 1940, carp and northern pike were introduced into the diet as about 50% of the total ration. The remainder of the diet was now 30% rabbit and 20% commercial feed. About 1 week after fish was added, some of the foxes

began to leave part of their feed. The number increased during the following week until practically all the foxes were refusing food. Neurologic symptoms were noted January 24 or 25, and the first death occurred January 26. Fish was removed from the diet at that time and the previous diet of rabbit and commercial feed was resumed. Deaths continued to occur until January 30 when the owner informed us of the epidemic. We immediately went to the ranch to observe the outbreak and to institute treatment.

Three foxes found dead in the pens at the time of our arrival brought the total number of deaths to ten. One other animal, lying on its side unable to rise and apparently moribund was given an intraperitoneal injection of 50 mg. of thiamine hydrochloride and 50 cc. of a 10% glucose solution. Without further treatment, this fox recovered so rapidly that in 12 hours it was able to stand on its feet, although unable to walk without staggering. Subsequently, tube-feeding of eggs, liver juice, milk, water and small amounts of yeast were given, and within 1 week the fox seemed to be entirely normal. It remained in good health and was bred successfully a few weeks later.

At the time of our visit on January 30, five foxes, in addition to the one already referred to, were off feed but showed no definite neurologic symptoms. Each was given an injection of 20 mg. of thiamine hydrochloride subcutaneously and, although one of the foxes had not eaten for 5 days, neither it nor any of the other four developed further symptoms of Chastek paralysis.

On January 31 large amounts of liver (about 30% of the total diet), some yeast and eggs were added to the diet of rabbit meat and commercial feed, and the outbreak soon ceased. While the number of animals available for this therapeutic test of synthetic thiamine hydrochloride was insufficient to permit the simultaneous use of controls, the results of the injections were so favorable that we believe they provide significant confirmatory evidence that the deficiency is really one of vitamin B_1, as seems proved by other evidence.

An opportunity to study in detail the effect of fish in fox rations was provided by extensive feeding trials which were conducted on the three large Fromm Brothers, Nieman and Company ranches near Thiensville, Wisconsin. Following a limited trial of fresh fish in the ration of the foxes during the winter of 1934–35, the feeding of fish in various forms was extended to more than 6700 foxes. Beginning on November 20, 1935, fish in three forms—fresh fish, canned fish and fish meal —was added to the fox ration. About January 1, losses of two or three foxes a day began to occur. Deaths were scattered throughout all the ranches. A few deaths occurred almost daily until January 25, when the number increased to twenty-nine for the 3-day period from January 25 to 27. During the next 3 days, January 28 to 30, fatalities climbed to a total of eighty-three, averaging almost twenty-eight a day. Fish in all forms was eliminated from the diet on January 25. After reaching a high point late in January, the mortalities subsided rapidly to about five a day for a period of 2 weeks. Further losses of two or three foxes a day continued for another 2 weeks with the outbreak ending during the first days of March. In table 3 are listed the ingredients of the diets containing the three types of fish preparations, together with the number of foxes fed these diets and the losses resulting in each group. The fresh fish was Atlantic whiting and the canned fish, Pacific coast mackerel. The symptoms and pathologic findings in foxes dying during the outbreak were diagnostic for Chastek paralysis.

From the data presented it appears that canned fish is less active than is fresh fish in producing Chastek paralysis. However, this is not a certainty, since the species of fish fed in the fresh state was different from that which was canned.

Of about 1200 foxes fed a diet containing 3% fish meal, only 0.6% died. The fish meal used on these ranches is a product so concentrated that 3% used in the rations is considered as equal in nutritive value to about 20% of fresh fish. It is probable that the process of preparing fish meal reduces the ability of that substance to produce Chastek paralysis.

Effect of Chastek paralysis on developing embryos and nursing pups

Two fox farms in Wisconsin, the Swinghammer Fur Farm and the Howland-Daly Fox Ranch, experienced during the month of May, 1938, a destruction of a large proportion of their crop of fox pups from Chastek paralysis. Fish was added to the ration on the Swinghammer Fur Farm on April 5, and

TABLE 3

Losses associated with the different diets fed to foxes on three ranches near Thiensville, Wisconsin, in January, 1936

	FRESH FISH	CANNED FISH	FISH MEAL
Number of foxes fed	4260	1292	1194
Number of deaths	286	14	7
Loss, in percentage	6.7	1.1	0.6
Complete dietary schedule[1]			
Fish	18.73	13.44	3.11
Liver	1.77	1.72	2.17
Wheat cereal	8.11	9.07	9.35
Oatmeal	4.59	4.90	5.30
Carrots	7.07	7.36	7.17
Lettuce	2.83	2.94	2.49
Horse meat	52.44	56.90	65.80
Eggs	2.20	2.16	2.74
Cod liver oil	0.21	0.22	0.25
Wheat germ oil	0.14	0.11	0.12
Mineral	0.14	0.20	0.25
Dry milk	1.77	0.98	1.25
Total	100.00	100.00	100.00

[1] Amounts of foods in the diets are listed as percentages of the total ration.

on the Howland-Daly Ranch, on April 7. A detailed itemization of the diets used on these ranches is presented in table 4.

The population on the Swinghammer ranch before the outbreak of fish disease was eight-five adult foxes and 175 pups. On May 4 the disease first appeared when fox pups in ten litters were found to be afflicted. Many more developed the disease on May 5 and 6. On the latter date a diagnosis of Chastek paralysis was made and appropriate treatment instituted. Fish was removed from the diet and increased amounts

of fresh meat, liver, milk and supplements of vitamin B_1 were added to the ration. As a result, the occurrence of new cases was promptly stopped. However, within the short span of 4 days, sixty-two puppies from twenty-five litters had died, a mortality of 35% of all puppies known to be on the ranch at the time.

On the Howland-Daly Ranch only one pup was ill on May 5, but forty-eight, or 33% of all pups on the ranch, had died by May 7, when treatment similar to that employed on the Swinghammer ranch was instituted and stopped the outbreak.

TABLE 4

Diets which caused Chastek paralysis on the Swinghammer and Howland-Daly Fur Farms

	SWINGHAMMER FUR FARM	HOWLAND-DALY FOX RANCH
	%	%
Horse meat	34.6	38.1
Fish	16.5	5.4
Fish meal		4.3
Cod liver oil	0.7	2.2
Ground green bone		10.9
Gland meat		10.9
Prepared cereal	36.2	21.7
Carrots	9.0	6.5
Raisins	3.0	
Total	100.00	100.00

The effect of Chastek paralysis on pregnancy has been clearly shown in several outbreaks. Markedly decomposed fetuses are usually found in the uteri of pregnant females that have died from Chastek paralysis. It appears certain that the fetus dies several days before the adult fox succumbs. The outbreak of this disease on the fur farm near Grand Forks, North Dakota, occurred at a time when most of the female foxes were pregnant. Although 134 adult females remained alive after the outbreak and showed no residual symptoms, only fifteen of them raised their young. Similar experience on other ranches has shown that a nonfatal attack of Chastek paralysis will regularly cause the intrauterine death of developing fetuses.

DISCUSSION

Since the only factor of apparent epidemiologic significance which is common to all ranches where Chastek paralysis has occurred appears to be the feeding of fresh fish as 10% or more of the ration, and since experimental data substantiate the fact that an otherwise satisfactory ration will produce Chastek paralysis regularly in foxes if fish is added, we feel there can be little doubt that the cause of Chastek paralysis is the feeding of fresh fish. Probably more carp than any other species of fish is used on ranches in the Midwest, and most of the outbreaks of the disease observed by us have been on ranches where carp was fed. In one case it was clear that Chastek paralysis was caused by the feeding of Atlantic coast whiting. On another ranch the fish was reported as being "mullets or suckers from Lake Manitoba". A ranch in Iowa suffered from Chastek paralysis as a result of the feeding of quillbacks from the Cedar River. It is apparent that many diverse kinds of fish, including species from both salt and fresh water, may cause the disease. However, we have some evidence to indicate that certain species, such as fresh water herring from Lake Superior or Michigan, are not nearly so destructive as are carp and the others just mentioned. Experiments now in progress are designed to demonstrate which parts of the injurious fish produce Chastek paralysis and, further, to determine why some fish are less active than others in the production of the disease.

There is clinical and experimental evidence (Perla, '38) obtained from work with human beings and with rats, that if a lactating female subsists on a ration moderately deficient in vitamin B_1, symptoms of a B_1 avitaminosis may develop in the nursing young while the mother remains in apparent good health. The severity of outbreaks of Chastek paralysis observed among nursing pups on ranches where the morbidity and mortality among adult animals was minimal is evidently an analogous phenomenon. There are pathologic, as well as clinical, differences between adult and young foxes with Chastek paralysis which may represent the differences between

the acute and the subacute or chronic disease. To what extent the factor of age, irrespective of acuteness or chronicity, influences the clinical and pathologic manifestation of the disease, we cannot say at this time.

Mechanism by which feeding of fish causes a vitamin-B_1 deficiency

Reported estimates of the amounts of vitamin B_1 found in various kinds of fish and red meats vary from 5 to several hundred international units per 100 gm., depending upon the kind of fish or meat and upon many experimental factors. It is apparent, however, that the expected decrease in the amount of this vitamin resulting from substituting fish for horse meat as 10%, or even as 25%, of the rations fed to foxes would not be enough to cause a B_1 avitaminosis. A small amount of fish may cause a vitamin-B_1 deficiency even though the diet, exclusive of fish, contains a moderate excess of that vitamin.

How fish is able to produce this result we do not yet know with certainty, but there are at least three possibilities. First, fish may somehow increase the animals' need for vitamin B_1 by a metabolic effect; second, fish may prevent absorption of the vitamin; and third, fish may bring about an inactivation or destruction of the vitamin in the food.

The present evidence is compatible with any of these explanations and the third one seems to us to be especially worthy of careful consideration. It appears reasonable to hypothesize that fish may inactivate or destroy the vitamin, if one postulates that small amounts of thiamine (1 mg. or less) found in a normal fox's daily ration may be destroyed, but that if relatively large doses are given, enough remains undestroyed to meet the needs of the fox. It is known that neutral sulfite readily splits thiamine. R. R. Williams ('39), after mentioning this fact, goes on to state: "Barium nitrite or sodium acetate appear, under certain conditions, to effect splitting in a similar sense and *it seems probable that, in neutral foods, free thiamin is very subject to corresponding*

splitting under influences which are as yet imperfectly understood.''[5] It may be that some constituent of fish acts as sodium sulfite, barium nitrite or sodium acetate do to inactivate thiamine.

In no case have we observed an outbreak of Chastek paralysis on a ranch where only cooked fish was fed. Apparently, that substance in fish which causes a B_1-avitaminosis in foxes is readily destroyed by heat.

Many of the early descriptions of beriberi were of cases among people in the Dutch East Indies and Japan whose diets consisted largely of polished rice and fish. Possibly the fish, as well as the rice, eaten by these persons may have been a factor in causing the deficiency disease which they developed. It is generally true in regions where fish is abundant that a variety of preparations using pickled or raw fish is commonly eaten.

Cleavage of thiamine by sodium sulfite, sodium acetate, or barium nitrite does not occur in a medium with a high acidity (Williams, '39) and probably the pH of the medium would exert a similar effect if the vitamin were inactivated by foods such as fish. This fact suggests that achlorhydria may influence the production of B_1 avitaminoses in man and lower animals. Obviously, most human patients with achlorhydria do not develop symptoms of B_1 avitaminosis, but in those on a limited diet containing an amount of vitamin B_1 barely sufficient for their needs, the lack of normal gastric acidity may permit destruction of enough of that vitamin to be of clinical importance. Clinical evidence suggests that achlorhydria is a factor in the production of Wernicke's disease.

SUMMARY

Chastek paralysis is an economically important acute disease of foxes occurring in violent outbreaks on ranches where uncooked fish is included as 10% or more of the ration. It causes a high mortality among nursing pups and intrauterine

[5] Italics by present authors.

death of fetuses. The disease may easily be produced experimentally with a diet containing fresh carp, and can be prevented by adding large amounts of thiamine to the same ration. Characteristic neurologic symptoms occur and diagnostic lesions, identical with those of Wernicke's hemorrhagic polioencephalitis of man, are found in the brain. Pathologic findings, limited therapeutic field trials of thiamine and histories of ranch outbreaks confirm the experimental evidence that Chastek paralysis is fundamentally a B_1 avitaminosis.

How fish can induce a deficiency of vitamin B_1 is unknown, but it is suggested there may be a chemical splitting of thiamine by some constituent of fish.

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THE SPECIFIC DYNAMIC EFFECTS OF AMINO ACIDS AND THEIR BEARING ON THE CAUSES OF SPECIFIC DYNAMIC EFFECTS OF PROTEINS [1]

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Since Lusk ('10, '12) first published his experiments on the influence of ingestion of amino acids on metabolism this subject received the attention of many investigators, and the experimental results obtained were often variously interpreted as explanations of the causes of specific dynamic effects of proteins. Some idea as to the complexity of the problem and of the scope and volume of the work accomplished may be obtained from the reviews of the literature by Lusk ('31 a), Grafe ('16), Mitchell and Hamilton ('29), Lundsgaard ('31), Borsook ('36), Brody and Procter ('33) and Wilhelmj ('35).

In spite of these extensive researches considerable confusion exists as to the specific dynamic values of the different amino acids and as to their bearing on the causes of specific dynamic effects of protein. This confusion of ideas, which is due mainly to the diversity of experimental results, is epitomized in the following words of Lusk ('28): "We may now proceed in greater detail into the clouded area of the cause of the specific dynamic action of protein. . . . The hypotheses which have been presented cannot now be welded into a concordant whole. They transcend one's powers to coordinate them."

[1] Authorized for publication on August 17, 1940, as paper no. 985 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

The following few specific references may serve to illustrate the diverse character of the experimental results and of their interpretations.

Thus, when Lusk ('12) found that glutamic acid administered to a dog was without effect on the heat production while the amino acid itself was metabolized, he concluded that the process of deaminization and urea formation must be ruled out from being a cause of the specific dynamic effect of protein. These observations and conclusions were later confirmed by Chambers and Lusk ('30). Grafe ('16), however, found that after the administration of glutamic acid to a dog the oxygen consumption was appreciably increased, and on the basis of his experiments with this and other amino acids he arrived at the conclusion that the specific dynamic effect of protein was largely due to the liberation of the NH_2 radicle of amino acids.

Glycine and alanine were found by Lusk ('12, '15) to exert pronounced effects on metabolism, the increase in heat production caused by glycine being considerably greater than that caused by alanine. These results were originally viewed by Lusk as support for his theory of cell stimulation. Aubel ('28) explained the specific dynamic effect of alanine by the transformation of this substance to glucose, and very recently Oberdisse ('39) concluded from his experiments with alanine administered to dogs intravenously that the specific dynamic effects of amino acids were due almost entirely to the nitrogen-free deamination products which are oxidized after conversion into carbohydrate.

Aspartic acid and asparagine showed no specific dynamic effect in the experiments of Atkinson and Lusk ('18); and the same significance was attached by the authors to this finding as to the negative results obtained with glutamic acid.

Lundsgaard ('31) observed that glycine, alanine, glutamic acid, aspartic acid and tyrosine all produced dynamic effects, and that these effects were in definite relationship with the amounts of nitrogen of administered amino acids.

Lusk's ('31 b) final view on this subject was in conformity with Rubner's ('02) general hypothesis that the specific dynamic effect of protein is due to the free heat of intermediary thermochemical reactions.

Viewing the available evidence as a whole one can hardly escape the conclusion that the question as to whether or not amino acids have definite specific dynamic values still remains unsettled. The numerous diverse data already on record seem to hold little promise that any further investigation of this subject, by the procedures thus far followed, will yield results of general uniformity and significance.

In the interest of further advance in this field of research new angles of approach to the problem of evaluating the dynamic effects of amino acids must be sought with a view of eliminating possible disturbing factors. This involves the question of procedure. The experiments presented herein represent an effort in this direction.

More specifically, this paper reports determinations of the specific dynamic effects of the amino acids glycine, alanine, glutamic acid, aspartic acid, asparagine and tyrosine by a procedure which constitutes a radical departure from the methods generally followed in the past work with amino acids.

DISCUSSION OF METHODS

The method employed in this investigation for the determination of the specific dynamic effects of the amino acids is an adaptation of the method developed by Kellner ('00) and by Armsby (Armsby and Fries, '03) for measuring the energy metabolism in relation to the diet. Most, if not all, of the past determinations of the specific dynamic values of amino acids reported in the literature were based on Rubner's method of measuring the specific dynamic effects of nutrients. There are at least two important differences between these two classical methods which need emphasis:

First, according to Rubner's procedure the test substance is administered only once and the heat measurement is begun soon after the substance is administered. If the period of

observation is short, as it was in most of the published experiments with amino acids, there is always a question as to what fraction of the ingested material is represented by the observed increase in heat production. According to the method of Kellner and of Armsby the heat measurement is not begun until after the animal has been on the experimental diet for a number of days, and thus it is likely to be well representative of the diet.

The other important difference between the methods is in the base values used for the determination of heat increments (dynamic effects) of feeding. The fundamental characteristic feature of Rubner's method is the use of the heat production of fast as the base value. The method used by Kellner features maintenance metabolism as basal. Armsby determined heat increments of feeding between various planes of nutrition, including submaintenance planes. It is, however, a significant fact that in a long series of calorimetric experiments, which were last planned by Armsby (see Fries, Braman and Cochrane, '24; Cochrane, Fries and Braman, '25), the heat production of maintenance was prominent as a base.

A question of crucial importance in the use of Rubner's method is whether the body nutrients katabolized during fast do not themselves exert dynamic effects. This question received relatively little attention in the past, a negative answer, that is, the non-existence of dynamic effects of body nutrients, being quite generally tacitly assumed. The significance of an affirmative answer to the foregoing question would be to the effect that the increase in heat production over the fasting metabolism, resulting from food consumption, is not to be taken as a measure of the true or absolute dynamic value of the food nutrients ingested, but as representing the difference between the dynamic effects of the ingested food nutrients and those of the body nutrients spared.

From the nature of the problem no direct proof of the existence or non-existence of dynamic effects of katabolized body nutrients seems possible. There is, however, sufficient indirect affirmative evidence in the literature to justify the considera-

tion of dynamic effects of body nutrients as a strong possibility, if not an established fact. Very briefly the evidence is along the following lines:

1. Increase in heat production accompanying an increase in protein break-down during fast (Rubner, '02; Chambers and Lusk, '30; Dann, Chambers and Lusk, '31).

2. The very low and much more variable heat increment values of rations and single nutrients below maintenance, with fasting heat production as a base, and with concomitant sparing of body nutrients, as compared with the higher and less variable heat increments caused by the addition of the same food substances to a maintenance ration (Richardson and Mason, '23; Forbes, Braman and Kriss, '28, '30; Kriss, Forbes and Miller, '34; Kriss, '38).

3. Thermochemical considerations. If the intermediary reactions and energy exchanges in the metabolism of food nutrients are accompanied by increased heat production (Adams, '26; Aubel, '28; Borsook and Winegarden, '31; Lusk, '31 b, and others), it is hardly conceivable that all the transformations incident to the katabolism of body nutrients during fast should be accomplished without energy expenditure. According to Borsook ('36) "there is no reason to expect different energy changes whether tissue protein and amino acids or amino acids immediately derived from ingested protein are metabolized."

4. Similarity between heat increment values of protein (casein), carbohydrate (starch) and fat (olive oil) determined with respect to fasting heat production as a base but corrected, by a conventional procedure, for the sparing of body nutrients, and the heat increment values of the same food nutrients determined by the use of the heat production of maintenance as a base (Kriss, Forbes and Miller, '34).

In view of all the foregoing considerations we feel justified in our departure from the use of the fasting heat production as a base value in our present study of the specific dynamic effects of amino acids, and in our attempt to follow the method embodying the principle of Kellner and Armsby, as elaborated

by Kriss, Forbes and Miller ('34) and as applied more recently to the determination of specific dynamic effects of different proteins (Kriss, '38).

EXPERIMENTAL PROCEDURE

The procedure followed in this investigation was, in most respects, the same as that used previously in the study of the specific dynamic effects of proteins (Kriss, '38). The experimental subjects were thirty-two male rats weighing approximately 200 gm. each. The same rats served as the subjects in previously reported studies of nitrogen, carbon and energy balances (Kriss, '39; Kriss and Marcy, '40 a), which were carried out in connection with and as part of the present study.

All rats received as their first experimental dietary treatment 8 gm. per day of a basal ration (as in the studies last cited) which was adequate for the maintenance of approximate equilibrium of nitrogen and energy. As in the previous experiments, the daily allowance of food was given in two equal portions, one in the morning and one in the evening. At the end of approximately 3 weeks on this diet the rats were subjected to respiration trials for the measurement of the total energy metabolism.

Following this treatment the rats, divided into groups, received, in addition to the basal ration, supplementary portions of different amino acids as the test substances. It was intended to feed all the supplements in quantities supplying 7500 calories of gross energy per day. Some of the amino acids, however, could not be consumed by the rats in such large quantities, and, consequently, were given in smaller amounts. The number of rats in each group, and the kind and quantity of supplement fed to each rat were as follows: Five rats received 2.06 gm. per day of d-glutamic acid. Six received 1.74 gm. of dl-alanine. Five received 1.8 gm. of glycine; one of this group (rat 11) received, in a different period, 2.4 gm. of glycine. Six received 1.288 gm. of l-tyrosine.

Five received 2 gm. of l-aspartic acid. Five received 2 gm. of l-asparagine.

The amino-acid-supplemented diets were fed to the rats for a period of 8 days. At the end of this period the rats were again subjected to the measurement of the total respiratory exchange. This was followed by a 7-day feeding period on the basal ration and another respiration experiment at the end of this period. Finally, after continued feeding on the basal ration for 7 days the rats were subjected to a 24-hour fast and to the determination of the fasting heat production in a 6- to 7-hour period. These determinations were used as a basis for correcting the total heat production of the animals for differences in body weight.

The respiratory measurements in the feeding periods began soon after the morning meal was consumed and continued for 7 consecutive hours. These measurements were used as a basis for the comparisons between the heat production of maintenance and supermaintenance feeding.

The apparatus used for measuring the respiratory exchange, and the general technic employed were as described in the previously cited publication (Kriss, '38).

The metabolism of protein and of amino acids was accounted for in the computation of the total heat production in the feeding periods on the basis of the urinary nitrogen excretions and the respiration and energy factors reported by Kriss ('39) and Kriss and Marcy ('40 a).

The practice of computing the heat production, for comparative purposes, to a basis of uniform empty body weight was continued. To this end the alimentary fill was determined, with each dietary treatment, in control animals which were comparable in weight with the experimental subjects.

DISCUSSION OF RESULTS

In the discussion of the results of the present experiments comparisons are made of certain of our data with results obtained by other investigators. These comparisons often bring out certain conflicting ideas. In considering these con-

trasts the reader should not lose sight of the differences in experimental procedure which were pointed out in the early part of the paper.

The data representing the average hourly heat production of the individual rats, per 200 gm. of empty body weight, and the calculated increases in heat production resulting from the addition of the various amino acid supplements to the basal ration are represented in table 1.

The uniformity of the heat production representing the basal ration is noteworthy. The average results of each group show only slight differences in metabolism between the initial and final maintenance periods. In accord with previous practice, the average heat production for the two maintenance periods (one immediately preceding and one immediately following the supermaintenance period) was used as the base value for the computation of the heat increments due to the supplements. It is believed that this procedure satisfactorily compensates for any slight changes in the metabolism incidental to the changes in age and body weight.

The heat production representing the amino acid-supplemented rations also shows a fair degree of uniformity within each group, and, without a single exception, is found to be higher than the corresponding value for the basal ration.

The increases in heat production of the individual rats, resulting from the administration of the amino acids, are presented in the last two columns of table 1 and are expressed as calories per hour and as per cent of metabolizable energy. The last-mentioned data served as the basis for the determination of the significance of differences between the specific dynamic effects of the different amino acids, which will be discussed later. The metabolizability of the amino acids was determined for each rat on the basis of the work reported by Kriss ('39) and by Kriss and Marcy ('40 a).

The variability of the results for heat production and for heat increments, within each group, is indicated by the respective values for standard deviation, which are self-explanatory. For the purpose of comparing the dynamic effects

TABLE 1

Hourly heat production of rats per 200 gm. of empty body weight as influenced by the addition of amino acids to a basal maintenance ration

RAT NO.	BASAL RATION, 8.0 GM. DAILY			BASAL RATION PLUS AMINO-ACID SUPPLEMENT	INCREASE IN HEAT PRODUCTION DUE TO AMINO ACID	
	Initial period	Final period	Average of the two periods	2.06 gm. glutamic acid	Total	Per cent of metabolizable energy
	cal.	*cal.*	*cal.*	*cal.*	*cal.*	
1	820	868	844	1020	176	71.3
2	854	875	865	1020	155	60.2
3	848	816	832	1015	183	81.3
4	801	825	813	990	177	67.1
5	787	781	784	955	171	62.5
Average	822	833	828	1000	172	67.9
Standard deviation	±28.7	±38.9	±30.8	±28.1	±10.6	±8.3
				2.4 gm. glycine		
11	813	886	850	973	123	61.5
				1.8 gm. glycine		
11	813	886	850	938	88	55.4
12	788	835	812	926	114	71.3
13	906	860	883	965	82	53.3
14	829	825	827	890	63	40.9
15	849	810	830	913	83	52.2
Average	837	843	840	926	86	54.8
Standard deviation	±44.6	±27.1	±27.4	±28.0	±18.2	±10.9
				1.74 gm. alanine		
21	829	805	817	939	122	59.9
22	841	785	813	947	134	66.4
23	821	791	806	969	163	77.9
24	830	797	814	932	118	55.3
25	878	838	858	980	122	61.4
26	894	810	852	946	92	42.4
Average	849	804	827	952	125	60.3
Standard deviation	±32.5	±18.1	±22.3	±18.5	±23.2	±13.6
				1.288 gm. tyrosine		
41	857	815	836	923	87	51.1
42	888	833	861	976	115	58.4
43	890	829	860	938	78	44.9
44	877	804	841	917	76	41.1
45	861	828	845	950	105	55.1
46	932	886	909	995	86	44.6
Average	884	833	859	950	91	49.2
Standard deviation	±27.1	±28.3	±26.7	±30.5	±15.5	±6.8
				2.0 gm. aspartic acid		
51	882	887	885	950	65	32.9
52	905	872	889	958	69	34.1
53	938	890	914	981	67	37.2
54	908	873	891	985	94	59.2
55	863	823	843	940	97	53.4
Average	899	869	884	963	79	42.4
Standard deviation	±28.4	±27.0	±25.8	±19.6	±15.7	±12.1
				2.0 gm. asparagine		
56	892	845	869	962	93	53.7
57	884	863	874	986	112	67.5
58	882	854	868	941	73	51.1
59	953	921	937	999	62	37.1
60	851	850	851	939	88	51.2
Average	892	867	880	965	85	51.8
Standard deviation	±37.3	±31.1	±33.1	±26.7	±19.2	±10.8

of the different amino acids, the average heat increment of each group was used.

The administration of glutamic acid resulted in pronounced and relatively uniform increases in heat production. These results are in contrast to the negative findings of Lusk ('12) and Chambers and Lusk ('30), and substantiate the positive dynamic effects of this amino acid observed by other investigators (Grafe, '16; Lundsgaard, '31; Luck and Lewis, '34).

Borsook ('36) suggested that the probable reason for Lusk's failure to observe any specific dynamic effect with glutamic acid was the short period of observation and the slow absorption of this amino acid from the intestine. In the present experiments glutamic acid constituted a part of the animals' diet for a period of 8 days and it was fairly completely absorbed (about 95%). In the light of the previous discussion of the question of technique, these experiments leave little doubt that the positive heat increments possess the greater significance.

Of similar significance are the positive increases in heat production caused by the administration of aspartic acid and asparagine. It will be recalled that these substances exhibited no significant dynamic effects in the experiments of Atkinson and Lusk ('18).

Glycine and alanine, administered in approximately equicaloric quantities, caused approximately the same hourly increases in heat production. The heat increments of equicaloric quantities of tyrosine were smaller. Tyrosine was less completely metabolized than were the other amino acids.

Different quantities of glycine supplement (2.4 gm. and 1.8 gm. per day) fed in different periods to the same animal (rat 11), produced nearly proportionate increases in heat production (51 calories and 49 calories per gram, respectively). This is interesting, since, in similar experiments with proteins (casein, gelatin and beef muscle) reported by Kriss ('38) nearly proportionate increases in heat production were observed when these substances were fed in quantities of 1.5 gm. and 3.0 gm. as supplements to a basal ration.

There is also some evidence (Forbes, Braman and Kriss, '28, '30) to the effect that the heat increments of mixed rations, above the maintenance level, are approximately proportional to the increases in food consumption within fairly wide limits.

TABLE 2

Specific dynamic effects of amino acids expressed in various ways

CATEGORY OF INTEREST	DAILY SUPPLEMENT							
	Glutamic acid 2.06 gm.	Alanine 1.74 gm.	Glycine 2.4 gm.	Glycine 1.8 gm.	Tyrosine 1.288 gm.	Aspartic acid 2.0 gm.	Asparagine 2.0 gm.	Coefficient of variation in per cent
Number of animals	5	6	1	5	6	5	5	
Metabolizable energy (Cal.)	6.08	4.98	4.80	3.77	4.44	4.47	3.94	
Specific dynamic effects (Cal.):								
Per 24 hours	4.13	3.00	2.95	2.06	2.18	1.90	2.04	
Per gram amino acid fed	2.00	1.72	1.23	1.15	1.70	0.95	1.02	29.2
Per gram nitrogen fed	21.50	11.11	6.66	6.22	22.29	9.16	5.57	60.8
Per gram extra urinary nitrogen	28.87	13.27	7.10	6.88	19.16	13.08	6.71	60.1
Per gram amino acid metabolized	2.83	2.91	1.44	1.37	2.73	1.43	1.46	37.2
Per millimol amino acid metabolized	0.42	0.26	0.11	0.10	0.49	0.19	0.19	59.8
Per gram of carbon of amino acid metabolized	6.93	7.19	4.49	4.26	4.57	3.95	4.01	27.5
Per 100 Calories of amino acid metabolized	92.3	82.0	69.1	65.1	49.2	63.4	67.1	19.9
Per 100 Calories of gross energy	54.9	39.6	39.7	37.0	29.1	32.8	33.9	21.8
Per 100 Calories of metabolizable energy	67.9	60.3	61.5	54.8	49.2	42.4	51.8	15.4

Since much of the explanation of the causes of specific dynamic action of proteins logically depends on the specific dynamic effects exhibited by different amino acids, the manner of expressing these effects obviously plays an important role.

With this in mind an attempt was made to bring out certain important relationships by expressing the results of these experiments in several different ways as presented in table 2.

The specific dynamic values expressed as Calories per gram of amino acid fed or as Calories per gram of amino acid metabolized show considerable variability. This is entirely expected in consideration of the different chemical structure and different metabolic paths of these substances. On either basis the dynamic values for glutamic acid, alanine and tyrosine are considerably higher than the corresponding values for glycine, aspartic acid and asparagine. The dynamic values for the latter two substances are nearly identical in spite of the marked difference in their nitrogen content.

Lack of a definite direct correlation between the specific dynamic effects of the amino acids and nitrogen metabolism is strikingly shown by the results expressed as Calories per gram of nitrogen fed, as Calories per millimol of amino acid metabolized, and especially, by the values expressed as Calories per gram of extra urinary nitrogen. All these three sets of values are characterized by very high coefficients of variation (about 60%). An inverse relationship is found to exist between the specific dynamic values and the nitrogen content of the amino acids in contrast to the direct correlation between these values observed by Lundsgaard ('31) for glycine, alanine, tyrosine, glutamic acid and aspartic acid. Obviously these methods of expressing the specific dynamic effects of amino acids do not contribute to uniformity. The results suggest that nitrogen metabolism and excretion apparently play a relatively insignificant role in causing the dynamic effects of amino acids.

It is probably significant that the dynamic effects are much more closely correlated with the carbon of the amino acid metabolized than with the extra nitrogen excreted in the urine. The dynamic values expressed per gram of urinary nitrogen vary from 6.71 Calories to 28.87 Calories; while those

expressed per gram of carbon metabolized vary only from 3.95 Calories to 7.19 Calories. The coefficient of variation of the former is 60.1% as compared with the coefficient of variation of 27.5% for the latter.

The figures for the dynamic effects per gram of carbon metabolized are smaller than the corresponding values per gram of urinary nitrogen. This difference in magnitude between the two sets of data is accounted for by the fact that the carbon content of the amino acids is several times their nitrogen content. It can in no way be taken to mean that the increase in heat production arising from the metabolism of carbon is less than that caused by the metabolism and excretion of nitrogen.

Borsook ('36) recently put forth the theory that "the specific dynamic effect of protein is a composite of two factors, one nearly constant, representing the increased energy production attending the metabolism and excretion of the nitrogen and amounts to 7 to 10 Calories per gram of nitrogen; the other —more variable, and at times larger fraction—arises from the metabolism of carbon." The results of the present investigation support the view that the latter factor is the much more important one of the two in this relation. The data do not preclude the possibility that metabolism of nitrogen (deaminization and urea formation) contributes some fraction to the increase in energy production; but they indicate that with rats this fraction is relatively very small—probably considerably smaller than Borsook's estimate. The total specific dynamic effect of glycine—the amino acid containing the highest percentage of nitrogen—is approximately 7 Calories per gram of extra urinary nitrogen. This value includes the increase in heat production arising from the metabolism of carbon. Very recently Kriss and Marcy ('40 b) reported evidence to the effect that excretion of nitrogen in the form of urea is not accompanied by appreciable expenditure of energy.

A further noticeable gain in uniformity of the dynamic values of the different amino acids is shown by the results

expressed per 100 Calories of amino acid metabolized (coefficient of variation equals 19.9%). Apparently the relationship between the energy metabolized and the specific dynamic effects of the amino acids is a more significant one than is either relationship between grams of nitrogen or grams of carbon metabolized and increased heat production. Metabolized energy comprises all the chemical energy of the test substance which was transformed in the body into heat. This was computed by the use of the energy factors determined by Kriss ('39) and Kriss and Marcy ('40 a). Apparently the heat increment of the non-nitrogenous (deaminized) fraction of the amino acid molecule is higher than the heat increment of the nitrogenous fraction.

A comparison of the dynamic values expressed as per cent of the gross energy with those expressed as Calories per gram of nitrogen fed indicates that the increase in heat production is more closely related to the potential energy of the amino acids than to their nitrogen content.

The smallest variation is shown by the dynamic effects expressed as per cent of metabolizable energy (gross energy minus energy of feces and urine, as determined directly), given in the last column of table 2 (coefficient of variation equals 15.4%). Of the latter values, that for glutamic acid is highest and that for aspartic acid is lowest. The dynamic values for alanine and glycine fed in equicaloric quantities are practically identical, but for the smaller quantity of glycine the dynamic effect as per cent of metabolizable energy is somewhat lower than for the larger quantity. Tyrosine and asparagine show practically the same dynamic effects as per cent of metabolizable energy, while their dynamic values per gram of nitrogen fed or per millimol of amino acid metabolized show the greatest difference.

The degree of correlation between the specific dynamic effects of the amino acids and the various metabolic factors may be seen best from the following results of statistical treatment of the data:

Coefficient of correlation between S.D.E. and—(1) metabolizable energy = 0.94; (2) gross energy = 0.70; (3) metabolized energy = 0.68; (4) metabolized carbon = 0.49; (5) millimols of amino acid metabolized = —0.24; (6) nitrogen ingested = —0.53; (7) extra urinary nitrogen = —0.60.

In order to ascertain the statistical significance of the differences among the dynamic effects of the different amino acids, expressed as per cent of metabolizable energy, the values obtained for the different amino acids with the individual animals (table 1, last column) were compared and subjected to a statistical analysis. The results of this analysis have revealed that, of the fifteen possible comparisons made, only three showed differences which were statistically significant. These three comparisons are between glutamic acid, on the one hand, and aspartic acid, asparagine and tyrosine, respectively, on the other. The physiological significance even of these three differences is, however, not certain.

It appears, therefore, that the specific dynamic effects of various amino acids, determined by the use of the heat production of maintenance as a base value, are best correlated with the metabolizable energy of the administered amino acids.

The results are consistent with the theory that the dynamic effects of amino acids, and therefore of proteins, are by-products of intermediary chemical reactions (oxidative and synthetic) and energy changes, and they do not lend support to the idea that certain amino acids, or certain of their cleavage products act in the body as special metabolic stimulants in the pharmacodynamic sense.

SUMMARY AND CONCLUSIONS

The specific dynamic effects of glutamic acid, glycine, alanine, tyrosine, aspartic acid and asparagine were determined by feeding these amino acids to rats in considerable quantities (some equicaloric) as supplements to a basal maintenance ration. The increases in heat production due to the supplements were measured with respect to the heat production of maintenance as a base value.

All amino acids showed positive dynamic effects. The results were expressed in ten different ways, and a study was made of the relationships between the heat increments and the various metabolic factors.

The heat increments expressed in relation to ingested or urinary nitrogen, as well as the values expressed per millimol of amino acid metabolized, were found to be highly variable, and showed no direct correlation between the specific dynamic effects of amino acids and nitrogen metabolism.

The results expressed as Calories per gram of carbon of the amino acids metabolized were much more uniform than those expressed per gram of extra urinary nitrogen, thus indicating that the metabolism of carbon is probably a more important factor in the production of the specific dynamic effects than is the metabolism of nitrogen.

The closest correlation was found between the dynamic effects and the metabolizable energy of the amino acids.

The results are consistent with the theory that dynamic effects of amino acids, and therefore of proteins, are by-products of intermediary chemical reactions (oxidative and synthetic) and energy changes, and they do not lend support to the idea that certain amino acids or certain of their cleavage products act in the body as special metabolic stimulants in the pharmacodynamic sense.

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SOME OBSERVATIONS OF VITAMIN B_6 DEFICIENCY IN THE DOG[1]

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THREE FIGURES

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Relatively little is known about the nutritional significance of vitamin B_6 in species other than the rat, although recent reports indicate that it is an essential for certain other animals such as the chick (Hegsted et al., '39), pigeon (Carter and O'Brien, '39), dog (Fouts et al., '39) and pig (Chick and associates, '38). The present paper is a report of observations made on dogs allowed to subsist on an artificial diet planned at the time to be as far as possible deficient with respect to vitamin B_6.

EXPERIMENTAL PROCEDURE

Diet employed. The percentage composition of the basal mixture was as follows: vitamin-free casein[3] 25; sucrose 48; hydrogenated cottonseed oil[4] 20; bone ash 4; salt mixture[5] 3. In addition each dog received daily, per kilogram of body weight, 10 μg. thiamine chloride,[6] 25 μg. riboflavin,[7] and filtrate

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[3] Labco brand casein, obtained from the Borden Company, Bainbridge, New York.

[4] Crisco.

[5] Slightly modified Osborne-Mendel salt mixture described by Street and Cowgill ('39).

[6] From the Winthrop Chemical Co., New York, N. Y.

[7] Obtained from the S.M.A. Corp., Cleveland, Ohio.

factor concentrate equivalent to 0.4 gm. liver extract. Vitamins A and D were provided in a cod liver oil concentrate available in the form of tablets, two tablets per dog daily.[8] Our experiments were almost finished when the discovery of the potency of nicotinic acid against canine blacktongue was announced. However, we believe that our animals were receiving nicotinic acid because of the way we prepared our concentrate of the filtrate factor from liver powder,[9] and because of the observation that our first administration of the concentrate in amounts equivalent to 0.2 gm. liver extract per kilogram body weight daily resulted in symptoms suggestive of incipient blacktongue, which symptoms disappeared when the dosage of this concentrate was doubled. In addition to the supplements just mentioned each control animal received daily a vitamin B_6 concentrate in the amount of 0.5 cc. per kilogram of body weight.[10]

Methods. Eight adult mongrel dogs, from 4½ to 9 kg. in weight, were used. They may be classified into three groups according to their dietary regimens.

Group I. Four animals (dogs 31, 33, 37 and 43) were given the basal diet lacking vitamin B_6. Each was offered daily an amount of food furnishing 80 calories per kilogram of body weight. As will be described below, three of these animals were given vitamin B_6 concentrate for short periods, beginning on the 122nd to the 356th day of the experiment, in order to study the curative effect of vitamin B_6 on some of the deficiency symptoms that had developed.

[8] Kindly furnished by the White Laboratories, Newark, New Jersey.

[9] One kilogram of liver extract powder, obtained through the courtesy of Dr. H. W. Rhodehamel of the Lilly Research Laboratories, was dissolved in 7.5 liters of water, the solution brought to approximately pH 4.0 with hydrochloric acid, and then 500 gm. of fullers' earth added with stirring. The earth was removed by decantation and filtration, and the process repeated with three more treatments using 500 gm. portions of the earth each time.

[10] This was prepared from tikitiki (Street and Cowgill, '39) by adsorption on fullers' earth and elution with alkali, as described by Lepkovsky and co-workers ('36) and was brought to a final concentration such that 1 cc. represented 3 gm. of rice polish. We have found concentrates prepared in this way to be potent sources of vitamin B_6 by rat assay.

Group II. Two dogs (nos. 32 and 34) in this group served as inanition controls for dogs 31 and 33, respectively, of group I; each control was given daily the amount of the basal mixture eaten by its experimental partner the preceding day. Dog 38 served as an ad libitum control: it was given the full allotment of 80 calories of the basal mixture per kilogram daily. In addition to the basal diet the control animals were given 0.5 cc. per kilogram per day of the vitamin B_6 concentrate.

Group III. This group is represented by a single animal, dog 36, which served as an ad libitum control for 348 days, receiving 0.5 to 1.0 cc. per kilogram of the vitamin B_6 concentrate daily. Because the dog appeared to have a higher requirement for vitamin B_6 than the other controls, vitamin B_6 was withdrawn on the 348th day to see if deficiency symptoms would develop.

Each dog was given a vermifuge before being placed on the experiment. The animals were weighed weekly and examined frequently for any changes in behavior or appearance. In the latter part of the experiment, after abnormalities of gait had appeared in some of the subjects, all were taught to traverse an obstacle board and to tread on a revolving table, so that changes in neuromuscular control could be seen more readily.

Weekly hemoglobin analyses, by the method of Newcomer ('19), and red blood cell counts were made on all animals. Occasional determinations were made of the free and combined acid in the gastric juice after the stimulation of gastric secretion by histamine or by means of a 7% alcohol test meal, as described by Cheney ('28).

The experimental dogs were autopsied immediately after they had succumbed to nutritional deficiency; the control animals were chloroformed and autopsied when their experimental companions died. Sections of the brain, spinal cord, peripheral nerves, liver, spleen and kidney were preserved for histologic examination, using the same techniques and staining methods as in our previously reported study.

RESULTS

General behavior of the animals. Within 4 to 5 weeks the appetites of the four animals not receiving vitamin B_6 declined and became erratic. However, anorexia never became complete as it so often does, for example, in vitamin B_1 deficiency. Concomitant with the decreased food consumption, the body weight declined gradually. The losses for these four dogs at the lowest point of their weight curves were 25, 36, 36 and 44% respectively, of the original weight. The four control animals appeared to have excellent appetites, always eating their food allotments quickly. Although two of these dogs were restricted to the food intakes of their experimental companions, none of them lost as much weight as the latter animals. Their weight losses were 10, 13, 13 and 15% of their initial weights, respectively.

Intermittent diarrhea occurred in the dogs of group 1; infrequently there was blood in the feces. This diarrhea was most pronounced in dog 37, in which it was present during about one-fourth of the days of the experiment. Thus, in contrast to our observations of canine riboflavin deficiency (Street and Cowgill, '39) and of chronic vitamin B_1 deficiency,[11] diarrhea is a significant feature of vitamin B_6 deficiency in dogs. On the other hand, vomiting was rarely seen in this experiment; none of the dogs was observed to vomit more than once or twice throughout the entire experimental period. The control animals did not suffer from diarrhea.

Gait. Abnormalities of gait appeared in three of the animals in group 1; dog 43, which did not survive as long as the others, did not show this change. In the case of dog 31 it was observed on the 231st day that the hind legs were stiff and that the animal stood with the hind legs far apart ("wide base"). The condition soon became more marked, and the gait so uncertain that the animal would fall over when it attempted to shake itself. Dog 33 showed changes in gait of the same type which, however, were much less marked; this was first noticed on the

[11] Unpublished observations.

245th day as a stiffness of the hind legs. By the 312th day there was a swaying of the hind quarters in walking. Abnormal gait appeared in dog 37 on the 217th day as a slightly spastic, slightly swaying gait. This abnormality became very gradually more marked until it was rather striking by the 434th day. At this time there was slight stiffness of the hind legs, wide base, and an awkward "compass gait", the hind legs swinging out wide at each step.

These dogs were examined several times by Dr. James C. Fox, Jr. An illustration of the observations made in this connection is seen in the following extracts of the reports pertaining to dog 37.

Dog 37—314th day. "The behavior on the obstacle board is odd. There is overstepping with the hind legs and prolongation of balancing on the forepaws. The dog is stable on the turntable, or may slip a little more than normal. Balances well on the hind legs. The deep reflexes are all normal."

423rd day. "After watching the dog's behavior the definite impression was gained that he was unsteady and ataxic on standing, but not weak. His hind legs overthrow on the obstacle board. He is uneasy on the turntable, preferring not to tread. Does not tire or slump when standing on his hind legs. The deep reflexes are lively. *Impression:* Posterior column degeneration."

As the syndrome of vitamin B_6 deficiency advanced the dogs became quieter, and sometimes weak and reluctant to walk. The administration of vitamin B_6 in the form of a concentrate (see figs. 1, 2 and 3) always brought about a return to the usual activity within a few days. The appetites of the experimental dogs also increased more or less promptly following treatment with vitamin B_6 concentrate.[12] In one of the instances in which the authors' B_6 concentrate was given, the appetite returned to normal within 24 hours; in two other instances a few days were required for complete restoration of the appetite. In two cases in which Merck's B_6 concentrate was given the appetite became normal within 24 hours, in the third case within 48 hours. The administration of Lepkovsky's

[12] Two different vitamin B_6 concentrates were used, namely, that prepared by the authors and a preparation kindly sent to us by Dr. W. L. Sampson of the Merck Institute of Therapeutic Research. The latter concentrate was reported to be entirely free of thiamine, riboflavin and filtrate factor.

('38) crystalline factor 1 to one animal (fig. 3) did not lead to any clear effect on food consumption or the degree of activity.

In contrast to the quietness and abnormal gait of the experimental dogs, the control animals of group 2 remained lively and vigorous and free from neuromuscular defects.

Hemoglobin and erythrocyte studies. Weekly blood analyses disclosed a gradually developing microcytic, hypochromic anemia, which became severe in three of the experimental dogs. In the fourth experimental animal (dog 43) the hemoglobin had fallen from an initial value of 15 gm. per 100 cc. to

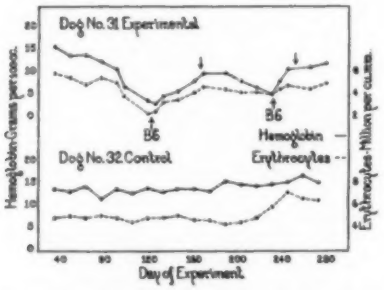

Fig. 1 Hemoglobin and erythrocyte levels of dog 31 and its inanition control mate. At the first arrow (↗ B_6) the administration of the authors' vitamin B_6 concentrate was begun at the level of 1.25 cc. daily. This was given for 31 days, followed by 2.50 cc. for 12 days. At the second arrow (↗ B_6) the Merck B_6 concentrate was given, fifteen single rat doses per day for 20 days. The inverted arrows (↙) indicate the withdrawal of the vitamin B_6 supplement.

9 gm. at the time of death. The hemoglobin and erythrocyte levels of the four control animals remained within the normal range with the exception that dog 36 showed a transitory anemia in the early part of the experiment, the hemoglobin falling to 6 gm. per 100 cc. The data for some of the animals are shown graphically in figures 1, 2 and 3. It will be observed that the hemoglobin of the animals of group 1 fell to levels of approximately 5 gm. per 100 cc. within from 120 to 320 days. When the anemia had reached this stage, treatment was instituted with vitamin B_6 preparation or, in one case, with ferrous sulfate. The B_6 concentrates and the ferrous sulfate were

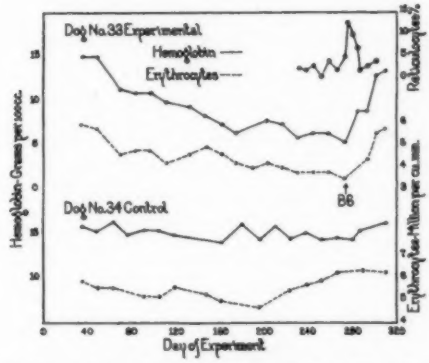

Fig. 2 Hemoglobin and erythrocyte levels of dog 33 and control mate. The arrow indicates the beginning of treatment with the Merck vitamin B_6 concentrate. Fifteen single rat doses were given daily for 20 days.

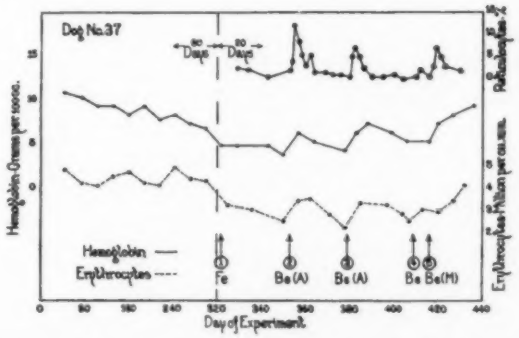

Fig. 3 Hemoglobin, erythrocyte and reticulocyte levels for dog 37. The first arrow indicates treatment with iron; 0.20 gm. of $FeSO_4 \cdot 7\,H_2O$ were given daily for 17 days, followed by 0.5 gm. daily for 7 days. At two the authors' vitamin B_6 concentrate was given, 5 cc. per day for 4 days. At three the same concentrate was given, 40 cc. in 4 days. The fourth arrow indicates the administration of Lepkovsky's crystalline factor 1 (vitamin B_6), 4 mg. given as the first dose, 2 mg. on each of the 3 following days. This material was given subcutaneously dissolved in distilled water. At five the Merck vitamin B_6 concentrate was administered, twenty single rat doses daily for 13 days.

given orally, but the crystalline factor 1[13] was administered subcutaneously. It will be observed that both vitamin B_6 concentrates caused a rapid increase in hemoglobin and erythrocyte levels. Reticulocyte peaks were reached in from 1 to 3 days in the four cases in which the reticulocyte curves were followed. The failure of the crystalline factor 1 (vitamin B_6) to influence the anemia of dog 37 is surprising, particularly since the animal responded in a uniformly satisfactory manner to vitamin B_6 concentrates. The dose of crystalline factor 1 given was approximately 500 μg. per kilogram of body weight daily, whereas Fouts, Helmer and Lepkovsky ('39) have found that the dose of 60 μg. of crystalline factor 1 per kilogram, given orally, effected a remission of the anemia appearing in adult dogs on a diet lacking vitamin B_6. This same animal, dog 37, responded satisfactorily to B_6 concentrate[14] given in an amount furnishing approximately 400 μg. of vitamin B_6 per kilogram (point 5, fig. 3), calculated on the assumption that a single rat unit of vitamin B_6 is 0.10 mg. No quantitative assays were made on the authors' vitamin B_6 concentrate used in this study. However, one of us (H.R.S.) at a later date determined that a vitamin B_6 concentrate made by this procedure contains about one-fourth the potency of the original tikitiki, so that on this basis our B_6 concentrate contained about 10 μg. of B_6 per cubic centimeter. It is evident, therefore, that this crude concentrate produced a satisfactory hematopoietic response in doses supplying relatively small amounts of vitamin B_6.

Two possible explanations for the inactivity of crystalline factor 1 suggest themselves. It is conceivable that the subcutaneous route of injection, by which the crystalline material was administered, is less effective than oral administration. On the other hand, it is possible that after this long period on the experimental diet (409 days) the animal lacked more than one essential hematopoietic factor, and that the vitamin

[13] We are indebted to Dr. Samuel Lepkovsky for a gift of his crystalline factor 1, or vitamin B_6 (Lepkovsky, '38).

[14] Merck's.

B_6 concentrate contained both or all of these, in contrast to the crystalline product.

It will be observed that large doses of ferrous sulfate given to dog 37 had absolutely no effect on the blood picture.

During the latter half of the experiment weekly leucocyte counts were made on all the animals. In contrast to the anemia observed in the animals of group 1, there was no tendency toward leucopenia, and no apparent difference between the leucocyte levels in the control group and those in the experimental group. Thus, in experimental dogs 31 and 33 the leucocyte counts varied from a minimum of 5100 to a maximum of 22,500 per cubic millimeter for the first animal, and from 5400 to 22,200 per cubic millimeter for the second. In the corresponding control animals, dogs 32 and 34, the leucocyte counts varied between 4300 and 14,900, and between 4100 and 7650, respectively.

Cardiac symptoms and pathology. Each of the animals in group 1 exhibited more or less marked symptoms of cardiac failure. These symptoms were most striking in dogs 31 and 33 and in both animals included dyspnea, tachycardia, large accumulations of serous fluid in the thorax, dilatation and hypertrophy of the right auricle and right ventricle, and chronic passive congestion of the liver. Dog 37 was more resistant to the shortcomings of the diet than the other animals in group 1, as indicated by the fact that it was the last to develop severe anemia; it never exhibited symptoms of cardiac embarrassment. However, when it was sacrificed on the 436th day there was found hypertrophy of both the right and left ventricles of the heart, more particularly of the right, and a slight dilatation of the right auricle. The heart was otherwise normal, as were the rest of the viscera. Sections of the liver showed a normal microscopic picture.

In contrast to the animals of group 1, the three controls of group 2 (dogs 32, 34 and 38) remained in excellent and vigorous health without the slightest suggestion of cardiac embarrassment. At autopsy their hearts looked entirely normal, as did the rest of the viscera.

The fourth control, dog 36, behaved anomalously throughout the experiment. As has already been mentioned, this animal served as an ad libitum control until the 348th day of the experiment when the vitamin B_6 concentrate was withdrawn in order to see whether symptoms of deficiency similar to those seen in the dogs of group 1 would appear. On the 369th day edema of the hind legs was observed, and 9 days later it was noticed that the abdomen was unusually full as though from ascites. By the 416th day distention of the abdomen had progressed to an alarming degree, and there was respiratory distress; the animal wheezed when placed on its back. When the dog was sacrificed on the following day, the thorax was found to be half full of clear serous fluid. Both the right ventricle and right auricle were greatly dilated and hypertrophied. The rest of the heart, including the valves, was normal. The abdomen was distended with several liters of clear fluid. There was marked edema everywhere; all the muscular tissues were jelly-like and dripping with fluid. The liver showed chronic passive congestion when examined histologically.

Peculiar attacks observed in dog 36. Shortly following the withdrawal of vitamin B_6 from the diet of this animal on the 348th day, the dog was observed to undergo periods of very transitory weakness and prostration. Six of these attacks were observed from the 352nd to the 361st day of the experiment, then no further were seen. In a typical attack, the animal would at first present a normal appearance, standing or walking in its cage, then suddenly it would begin to sway violently and then fall over. When picked up at this point it was completely limp and would make not the slightest effort to stand. About ¼ minute later the animal would get up and walk around in a normal manner as though nothing had happened. On one occasion the dog was observed to walk back and forth in its cage and jump up and down against the door in an excited manner 2 or 3 minutes before one of the attacks of weakness.

No seizures of this kind were ever seen in any of the other animals of this study. However, somewhat similar symptoms have been reported in pigs suffering from lack of vitamin B_6.

Chick and co-workers ('38) stated that five pigs on a diet lacking vitamin B_6 occasionally exhibited epileptic fits. According to their description, in these seizures the animal at first ran about wildly, then fell over as though shot, and became pale and comatose for a few minutes. Recovery was rapid, and the animal would resume its previous occupation. The attacks of weakness occurring in dog 36 are thus similar to those seen in pigs on a similar diet, if one assumes that the excitement phase is less pronounced in the dog.

TABLE 1

Myelin degeneration appearing on a diet lacking vitamin B_6

	LENGTH OF EXPERIMENTAL PERIOD	SCIATIC NERVE		SPINAL CORD		
		Marchi	Spielmeyer	Marchi	Spielmeyer	Nissl
	days					
Group 1						
Experimental						
Dog 31	355	4+	2+	3+	+	slight gliosis
Dog 33	317	2+	0	+	0	0
Dog 37	436	0	0	0	0	0
Dog 43	199	2+	0	0	0	0
Group 2						
Controls						
Dog 32	433	2+	0	0	0	0
Dog 34	319	3+	3+	0	0	0
Dog 38	319	2+	0	0	0	0
Group 3						
Dog 36	417	2+	+	±	0	0

Histopathology of the nervous system. Microscopic examination of the nervous system revealed degenerative changes in the myelin sheaths of certain of the animals; these are detailed in table 1. It will be observed that both experimental and control groups showed moderate to fairly severe degeneration of sciatic nerves. However, none of the three control animals exhibited any changes in the spinal cord, while two of the dogs in group 1 had myelin degeneration in the posterior columns and dog 36 showed slight degeneration of the posterior columns. There was no high degree of correlation

between the clinical symptoms of deranged neuromuscular function and the histologic findings. It is true that dog 31, with the most striking clinical symptoms showed the most severe myelin destruction, but on the other hand, the nervous system of dog 37 was essentially intact in microscopic appearance, yet this animal had developed a slight ataxia before the end of the experiment. This animal was the last to develop the characteristic symptoms of vitamin B_6 deficiency, presumably because of protection from vitamin deficiency through coprophagy. It was observed to eat its feces until it was muzzled in the latter part of the experiment.

The results of this study do not settle with any finality the role of vitamin B_6 in the functioning of the nervous system, since we did not succeed in entirely preventing myelin changes in the control group receiving vitamin B_6. However, we feel that the most probable explanation of our observations is that the amount of vitamin B_6 given to the controls (the equivalent of 1.5 gm. of rice polish per kilogram daily) while adequate to maintain them in apparently good health, was, after all, not quite enough to prevent early changes in the nervous system. This seems all the more probable, since this amount of vitamin B_6 was definitely too small to maintain health in dog 36.

Gastric juice analyses. Between the 265th and 342nd day of the experiment, several analyses of gastric juice were made on the seven surviving dogs. Following an overnight fast, 25 cc. of 7% alcohol were administered, and the stomach contents were removed as completely as possible 30 minutes later through a Reyfuss tube. The results are summarized in table 2. It will be observed that the volumes of the samples obtained from experimental dogs 31 and 33 were much smaller than those from the three control animals; also the average titer of free acid was about half that of the controls. Occasionally, samples were obtained from dogs 31 and 33 which contained no free acid. However, there was never a true achlorhydria, since stimulation with histamine in these two dogs promoted the secretion of juice containing normal amounts of free acid. In one of the animals there was an indication that the admin-

istration of vitamin B_6 concentrate might act to increase the free acid content of the gastric juice; in the case of dog 33, three determinations of free acid just prior to treatment with vitamin B_6 gave values of 9, 3 and 19, expressed as cubic centimeters of N/10 HCl per 100 cc. of gastric juice. Following treatment with the B_6 concentrate[15] (fig. 2) the next four determinations gave values of 37, 0, 48 and 58 cc. of free acid.

TABLE 2

Analyses of gastric juice samples obtained after stimulation with a 7% alcohol test meal

GROUP	DOG NO.	NUMBER OF DETERMINATIONS	AVERAGE VOLUME OF SAMPLES	AVERAGE VALUE FOR FREE ACID
			cc.	*cc. of N/10 acid per 100 cc.*
Experimental	31	5	7.2	26
	33	7	6.9	25
	37	6	15.7	45
Control	32	2	21.5	49
	34	2	15.0	47
	38	3	29.3	57

DISCUSSION

Our results confirm the findings of Fouts et al. ('38), using dogs, and Chick and associates ('38), using pigs, that a diet containing thiamine, riboflavin and filtrate factor, but not vitamin B_6, leads to the development of a microcytic anemia. Our inability to effect a cure of the anemia with crystalline vitamin B_6 would seem to suggest that other factors may be involved in the production of anemia with the particular diet that we have used. Further work is needed to clarify this point.

We believe that the finding in several of our animals of well-advanced dilatation and hypertrophy of the right side of the heart, with other symptoms of congestive heart failure, is a matter of some interest since, to our knowledge, such a condition has not been produced in nutrition studies except in vitamin B_1 deficiency. Lack of vitamin B_1 could hardly be a factor in the present study, since the animals received 10 μg. of vitamin B_1 per kilogram daily, whereas we have found

[15] See footnote 14, p. 282.

(Street, Zimmerman, Cowgill, Hoff and Fox, '41) that the daily administration of 7 to 9 μg. of vitamin B_1 per kilogram prevents the cardiac hypertrophy which occurs in dogs subsisting on a diet lacking vitamin B_1. The present study does not show whether or not the congestive heart failure was due specifically to lack of vitamin B_6, but since the condition was prevented by the administration of a vitamin B_6 concentrate, it would appear that the cardiac dysfunction was presumably caused either by lack of vitamin B_6 or some as yet unidentified factor occurring in B_6 concentrates. It is interesting to note that in dog 36, which suffered from only a moderate shortage of vitamin B_6, marked symptoms of cardiac embarrassment appeared at the close of the experiment, despite the fact that the appetite was good throughout most of the experimental period, and there was only moderate loss of weight (13%), while the behavior was essentially that of a normal animal. This would suggest that lack of a dietary essential may sometimes cause cardiac derangement in the absence of other obvious symptoms of inadequate nutrition.

Likewise, in the case of the myelin degeneration of the nervous system it is not possible to state that lack of vitamin B_6 is the etiological factor; it is clear, however, that some factor in the vitamin B_6 concentrate given the control dogs considerably reduced the severity of the pathological changes in the nervous system. With the exception of vitamin B_1, the role of the individual members of the vitamin B complex in preserving the integrity of the nervous system is largely unexplored. However, Wintrobe, Mitchell and Kolb ('38) have demonstrated that lack of members of the B group other than B_1 and riboflavin may cause myelin degeneration in pigs, and the present study indicates that vitamin B_6 may be one of the further factors essential for the maintenance of the nervous system.

The moderately severe myelin degeneration of the peripheral nerves observed in the three control dogs of group 2 after the conclusion of the study was surprising to us, since during the experiment these animals were lively, had good appetites and, in general, appeared to be in excellent health. This obser-

vation suggests that a dietary regime which maintains apparent health is not necessarily adequate for the preservation of the integrity of the nervous system over extended periods.

It is clear that there is a great difference in the form which vitamin B_6 deficiency takes in the dog as compared to the rat. Although severe dermatitis of the paws and nose is the chief symptom of this condition in the rat, in the adult dogs used in this study, there were no cutaneous symptoms whatever. There is also a striking difference between the picture of chronic disease that we have produced by lack of vitamin B_6 in adult dogs, and the very rapidly developing deficiency state which McKibben, Madden, Black and Elvehjem ('39) observed in puppies given a diet lacking vitamin B_6. In these puppies no definite, consistent deficiency symptoms were seen other than anemia, loss of weight, anorexia and death. Undoubtedly puppies are superior as assay animals in studying the preparation of vitamin B factors needed by the dog but, on the other hand, adult dogs are superior for studying the nature of the syndromes which one might expect to appear in man as a result of lack of the various B factors.

SUMMARY

Dogs maintained on a diet containing, of the B vitamins, thiamine, riboflavin, and filtrate factor, but not vitamin B_6, developed a severe anemia within 120 to 320 days. Treatment with vitamin B_6 concentrates led to a rapid increase of erythrocyte and hemoglobin levels, while treatment with ferrous sulfate produced no response.

After 300 days or more on the diet some of the animals developed marked symptoms of cardiac embarrassment, including dyspnea, tachycardia, dilatation and hypertrophy of the right ventricle and right auricle, accumulation of serous fluid in the thorax, and chronic passive congestion of the liver.

Degenerative changes were found in the myelin sheaths of the peripheral nerves and of the spinal cords of the experimental animals. These changes were of lighter degree in the control animals, receiving vitamin B_6 concentrate, and occurred only in the peripheral nerves.

Analyses of the gastric juice carried out in the latter part of the experiment gave a suggestion that in vitamin B_6 deficiency there may be a decrease in the secretion of acid and possibly in the volume of juice secreted.

Control animals receiving the same diet, except that they were given vitamin B_6 concentrate in addition, did not become anemic and showed no evidence of cardiac dysfunction either during life or at autopsy.

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CHOLINE METABOLISM

V. THE EFFECT OF SUPPLEMENTARY CHOLINE, METHIONINE AND CYSTINE AND OF CASEIN, LACTALBUMIN, FIBRIN, EDESTIN AND GELATIN IN HEMORRHAGIC DEGENERATION IN YOUNG RATS [1]

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THREE FIGURES

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This investigation is a continuation of previously reported observations of hemorrhagic degeneration in young rats (Griffith and Wade, '39, '40; Griffith, '40 a and '40 b) and is a study of the influence of methionine and of cystine on the requirement of choline. The occurrence and severity of hemorrhagic degeneration have been determined in rats fed twenty-five diets varying in protein content from 18 to 40%, in total methionine from 0.4 to 1.2% and in total cystine from 0.05 to 1.2%. The proteins, casein, lactalbumin, fibrin, edestin and gelatin were used with and without supplements of methionine and cystine. Variations in the degree of choline deficiency were measured by the severity of the characteristic renal hemorrhage, by the increase in the weight of the kidneys and by the extent of deposition of fat in the liver.

EXPERIMENTAL

The experimental procedures and the constituents of the diet were similar to those previously described (Griffith and Wade, '39). The appearance of the kidneys was noted at the

[1] Presented before the Division of Biological Chemistry of the American Chemical Society at Detroit, Mich., September 9 to 13, 1940.

end of the experimental period and the per cent of animals in each group showing the hemorrhagic state (Griffith, '40 b) is recorded in tables 1 to 4 and in figure 2 under the heading "renal lesions." Determinations were made of liver and kidney weights and of the total chloroform-soluble substances in the liver. The term "liver fat" in tables 2 to 4 and in figure 3 refers to this fraction.

Young male rats, 20 days of age and 25 to 34 gm. in weight, were used in the experiments recorded in table 1. The basal diet, AC 34, consisted of purified casein 15%, lard 9, sucrose 62.9, salt mixture 4 (Hawk and Oser, '31), calcium carbonate 1, agar 2, whole dried yeast 6, and the fortified fish liver oil,[2] 0.1%.

In the experiments listed in tables 2 to 4 young male rats, 21 to 26 days of age and 38 to 42 gm. in weight were employed. The basal diet was the same as AC 34 except that lard was increased to 20% and various protein levels and mixtures were used. All additions to the basal diet replaced an equal weight of sucrose. The composition of the protein fraction of each ration is indicated in tables 2 to 4. These tables also show the total methionine and cystine in each diet, exclusive of the yeast protein. The values for these sulphur-containing amino acids in casein, lactalbumin, fibrin, edestin and gelatin were taken from the data of Baernstein ('32, '34 and '36) and are as follows: methionine, 3.1, 2.3, 2.4, 2.2, 1.0% respectively and cystine, 0.3, 3.4, 1.7, 1.4, 0.5% respectively. Supplements of l-cystine and of dl-methionine were used in certain diets. Best and Ridout ('40) have found the racemic mixture as potent as the natural methionine in the prevention of fatty livers in older rats.

RESULTS

Relation of dietary cystine to hemorrhagic degeneration. Table 1 shows the occurrence of renal lesions and figure 1 the corresponding increase in kidney weight on diets containing 0, 0.3, 0.5 and 1.0% of added cystine. In addition, the intake

[2] Natola.

TABLE 1

Effect of choline and of cystine on hemorrhagic degeneration in young male rats [1]

GROUP	NUMBER OF RATS	FINAL BODY WEIGHT	FOOD PER DAY	CHOLINE CHLORIDE ADDED PER GRAM OF FOOD	CYSTINE ADDED PER GRAM OF FOOD	PER CENT OF RATS WITH RENAL LESIONS
		gm.	*gm.*	*mg.*	*mg.*	
A 1	40	43	4.6	0	0	80
A 2	40	45	5.0	0.125	0	30
A 3	25	46	5.4	0.250	0	4
B 4	39	40	3.4	0	3	92
B 5	49	45	4.4	0.125	3	76
B 6	36	47	4.9	0.250	3	45
B 7	22	52	5.3	0.375	3	0
C 8	30	42	4.0	0	5	93
C 9	40	47	4.5	0.125	5	80
C 10	20	49	4.7	0.250	5	70
C 11	17	49	4.8	0.375	5	0
C 12	12	52	5.2	0.50	5	0
D 13	30	40	3.9	0	10	100
D 14	46	44	4.1	0.125	10	83
D 15	34	48	4.7	0.250	10	41
D 16	25	49	5.0	0.500	10	0

[1] Experimental period—7 days. Average weight at start—29 gm. Age at start—20 days. Basal diet AC 34.

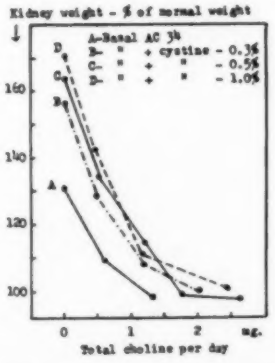

Fig. 1 The effect of choline and of cystine on the increase in kidney weight due to renal hemorrhagic degeneration. The occurrence of renal lesions in these groups is recorded in table 1.

of choline necessary to maintain the normal appearance and weight of the kidneys was determined for each level of added cystine. Although these results confirmed the previously reported injurious effect of cystine (Griffith and Wade, '40), it was evident that renal damage was not at all proportional to the amount of cystine ingested. The minimum daily requirement of choline for protection of the kidneys was the same regardless of whether the diet contained 0.3 or 1.0% of added cystine. However, the higher cystine levels were slightly more toxic in the absence of added choline. It would appear either that only a certain fraction of the dietary cystine was metabolized in such a way that extra choline was required or that variable quantities of cystine were normally metabolized in the presence of a fixed amount of choline. In these experiments cystine was not found to be a toxic amino acid if the diet contained an adequate supply of choline.

The effect of various proteins on hemorrhagic degeneration. Table 2 shows the relative effects of a low choline diet containing 18% of casein and of diets containing 12% of casein plus 6% of lactalbumin, fibrin, edestin or gelatin (groups 1 to 5). Substitution of part of the casein with these proteins did not prevent the effects of choline deficiency since, in each instance, deposition of liver fat was excessive and renal lesions were severe. These five diets were then compared after the addition of choline (groups 6 to 10, table 2) inasmuch as it has been our experience that significant differences which were masked on severe diets, might appear if the diets were supplemented with partially protective levels of choline. It was clearly evident from the results that casein was the most protective of the proteins used and that the effects of choline deficiency were aggravated by lactalbumin, fibrin, edestin and gelatin. The inclusion of these four proteins in diets produced food mixtures containing less methionine and more cystine, a shift which increased the requirement of choline (Griffith and Wade, '40).

The relation between the methionine content of the diet and hemorrhagic degeneration. Tables 3 and 4 show the varying

TABLE 2

The effect of dietary proteins on the deposition of liver fat and on the occurrence and severity of hemorrhagic degeneration in 40-gm. male rats after an 8-day experimental period

GROUP [1]	1	2	3	4	5	6	7	8	9	10
Choline chloride added per gram of food, mg.	0	0	0	0	0	0.2	0.2	0.2	0.2	0.2
Dietary protein casein, %	18	12	12	12	12	18	12	12	12	12
Lactalbumin, %	0	6	0	0	0	0	6	0	0	0
Fibrin, %	0	0	6	0	0	0	0	6	0	0
Edestin, %	0	0	0	6	0	0	0	0	6	0
Gelatin, %	0	0	0	0	6	0	0	0	0	6
Average final body weight, gm.	60	54	58	56	54	65	63	61	62	62
Average food consumption per day, gm.	5.1	4.4	4.6	4.4	4.6	5.5	5.1	4.9	5.3	5.4
Liver weight as per cent of body weight [2]	6.9	7.4	6.7	6.7	6.6	5.8	6.8	6.5	6.4	6.6
Average weight of liver fat, mg.	897	1009	990	895	895	536	1030	872	828	870
Liver fat as per cent of liver [3]	21.5	25.0	25.2	23.7	25.3	14.1	23.9	21.7	21.1	21.4
Average weight of kidneys (per pair), mg.	928	1002	954	955	935	731	790	811	772	811
Kidney weight as per cent of body weight [4]	1.54	1.85	1.63	1.71	1.73	1.13	1.25	1.32	1.25	1.31
Renal hemorrhage, %	81	86	86	95	86	0	48	48	52	43
Total methionine per gram of food, mg.	5.6	5.1	5.2	5.0	4.3	5.6	5.1	5.2	5.0	4.3
Total cystine per gram of food, mg.	0.5	2.4	1.4	1.2	0.7	0.5	2.4	1.4	1.2	0.7

[1] Each group consisted of 21 rats, 21 to 26 days of age and 38 to 42 gm. in weight.
[2] Normal per cent of body weight for rats in this colony, 4.31.
[3] Normal per cent of liver for rats in this colony, 4.0.
[4] Normal per cent of body weight for rats in this colony, 1.0 to 1.2.

TABLE 3

The effect of dietary proteins and of added methionine and cystine on the deposition of liver fat and on the occurrence and severity of hemorrhagic degeneration in 40 gm. male rats after an 8-day experimental period

GROUP [1]	11	12	13	14	15	16	17	18	19	20 [2]	21 [3]
Dietary protein											
Casein, %	12	0	18	18	40	40	18	18	18	18	18
Lactalbumin, %	0	16	22	0	0	0	0	0	0	0	0
Fibrin, %	12	16	0	22	0	0	0	0	0	0	0
Gelatin, %	0	0	0	0	0	0	22	22	22	22	22
Methionine added per gram of food, mg.	0	0	0	0	0	0	0	0	4	0	0
Cystine added per gram of food, mg.	0	0	3	3	3	10	3	0	10	10	0
Average final body weight, gm.	62	59	63	63	67	64	58	56	63	59	60
Average food consumption per day, gm.	5.4	5.0	5.3	5.1	5.2	4.7	4.7	4.2	4.7	4.5	4.5
Liver weight as per cent of body weight [4]	6.3	5.5	5.1	5.0	5.2	5.5	6.5	6.2	5.3	5.1	4.8
Average weight of liver fat, mg.	738	432	140	149	196	166	845	723	273	370	134
Liver fat as per cent of liver [5]	19.0	13.4	4.3	4.8	5.6	4.7	22.5	20.9	8.2	12.3	4.7
Average weight of kidneys (per pair), mg.	835	758	812	765	815	880	1080	1061	1070	1025	975
Kidney weight as per cent of body weight [6]	1.34	1.28	1.29	1.21	1.22	1.37	1.86	1.92	1.71	1.73	1.63
Renal hemorrhage, %	29	10	0	0	0	14	81	50	10	10	10
Total methionine per gram of food, mg.	6.6	7.5	10.6	10.9	12.4	12.4	7.8	7.8	11.8	7.8	7.8
Total cystine per gram of food, mg.	2.4	8.2	11.0	7.3	4.2	11.2	4.6	1.6	11.6	11.6	1.6

[1] Each group consisted of 21 rats, 21 to 26 days of age and 38 to 42 gm. in weight.
[2] 0.5 mg. of choline chloride added per gram of food.
[3] 2.0 mg. of choline chloride added per gram of food.
[4] Normal per cent of body weight for rats in this colony, 4.31.
[5] Normal per cent of liver for rats in this colony, 4.0.
[6] Normal per cent of body weight for rats in this colony, 1.0 to 1.2.

[4] Normal per cent of body weight for rats in this colony, 1.0 to 1.2.

TABLE 4

The effect of dietary proteins and of added methionine and cystine on the deposition of liver fat and on the occurrence and severity of hemorrhagic degeneration in 40-gm. male rats after an 8-day experimental period

GROUP [1]	22	23	24	25	26	27	28	29	30	31	32
Dietary protein											
Casein, %	18	18	18	18	18	9	9	9	9	9	0
Lactalbumin, %	0	0	0	0	0	3	3	3	3	3	9
Fibrin, %	0	0	0	0	0	3	3	3	3	3	9
Gelatin, %	0	0	0	0	0	3	3	3	3	3	0
Methionine added per gram of food, mg.	1	0	0	1	3	0	1	2	4	6	0
Cystine added per gram of food, mg.	0	1	3	3	3	3	3	3	3	3	0
Average final body weight, gm.	65	57	59	61	67	56	58	66	66	67	54
Average food consumption per day, gm.	5.4	4.6	4.6	4.8	5.5	4.4	4.6	5.3	5.4	5.3	....
Liver weight as per cent of body weight [2]	6.4	6.9	7.4	6.4	5.3	7.6	7.1	6.3	5.2	5.3	7.0
Average weight of liver fat, mg.	721	861	1115	885	284	1097	1142	973	274	201	1103
Liver fat as per cent of liver [3]	17.3	22.0	25.5	22.8	8.0	26.2	28.0	23.7	8.0	5.7	29.2
Average weight of kidneys (per pair), mg.	759	1028	1036	840	725	980	973	828	756	792	895
Kidney weight as per cent of body weight [4]	1.16	1.82	1.75	1.38	1.08	1.77	1.68	1.26	1.15	1.19	1.66
Renal hemorrhage, %	29	86	95	67	0	95	86	43	0	0	91
Total methionine per gram of food, mg.	6.6	5.6	5.6	6.6	8.6	4.5	5.5	6.5	8.5	10.5	4.2
Total cystine per gram of food, mg.	0.5	1.5	3.5	3.5	3.5	5.0	5.0	5.0	5.0	5.0	4.6

[1] Each group consisted of 21 rats, 21 to 26 days of age and 38 to 42 gm. in weight.
[2] Normal per cent of body weight for rats in this colony, 4.31.
[3] Normal per cent of liver for rats in this colony, 4.0.
[4] Normal per cent of body weight for rats in this colony, 1.0 to 1.2.

degrees of protection against choline deficiency on diets differing in total protein, methionine and cystine. It was previously shown by Griffith and Wade ('40) that a 40% level of casein in a ration was completely protective whereas 15 and 25% levels were injurious. The same defense against hemorrhagic degeneration was apparent on food mixtures containing 18% casein plus 22% lactalbumin or fibrin even though extra cystine was added (groups 13 to 15, table 3). Similar protection resulted from the addition of methionine to diets containing 18% protein (groups 26, 30 and 31, table 4).

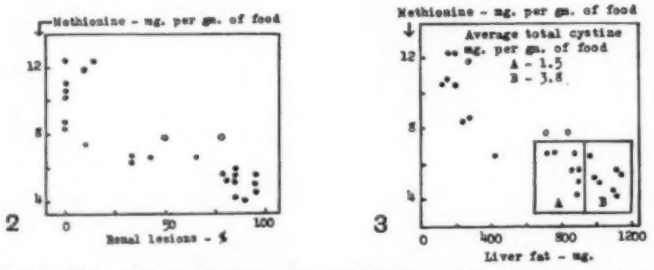

Fig. 2 The relation between the methionine content of the diet and the occurrence of renal lesions in the twenty-five groups of rats in tables 2, 3 and 4 which did not receive added choline. Diets containing 22% of gelatin are represented by circles.

Fig. 3 The relation between the methionine content of the diet and the deposition of liver fat in the twenty-five groups of rats in tables 2, 3 and 4 which did not receive added choline. Diets containing 22% of gelatin are represented by circles.

The fact that hemorrhagic degeneration was absent, or nearly so, in each group receiving more than 8 mg. of methionine per gram of food, indicated that dietary choline was not essential if the food mixture supplied sufficient methionine. This effect of methionine is clearly evident in figures 2 and 3 in which total methionine was plotted against renal lesions and liver fat, respectively, for the twenty-five different diets listed in tables 2, 3 and 4 which did not contain added choline. Choline appeared to be a dietary essential in these diets only if less than 8 mg. of methionine per gram of food was present. With less than 8 mg. the renal lesions and deposition of liver fat

occurred and varied according to the methionine and cystine levels. Group 16, table 3, was the only high methionine group exclusive of the high gelatin diets (groups 17 to 21, table 3) which failed to show complete protection against hemorrhagic degeneration. Only three of the twenty-one rats showed renal lesions and the livers were not fatty. It has been exceptional for renal hemorrhage to occur in the absence of a deposition of liver fat.

Anomalous results followed the feeding of diets containing 18% of casein and 22% of gelatin (groups 17 to 21, table 3). Although groups 17, 18 and 19 (shown by circles in figures 2 and 3) did not deviate greatly from the other groups and although the addition of extra methionine (group 19) and of choline (groups 20 and 21) decreased the deposition of liver fat and the occurrence of renal lesions, there was in every case a marked increase in the weight of the kidneys. Such an enlargement of the kidneys without the simultaneous occurrence of hemorrhagic degeneration has been observed in this investigation only on diets containing a large amount of gelatin.[3] The fact that these food mixtures also contained 18% of casein indicated that the effect of gelatin was positive in its nature. This finding confirmed the similar observation of Jackson, Sommer and Rose ('28) who noted severe renal pathology in rats fed a ration high in gelatin.

DISCUSSION

Our investigation of choline metabolism in young growing rats has been concerned primarily with the relation of choline to the maintenance of normal renal structures and only secondarily to the relation between choline and liver fat. The investigations of others (Best and Ridout, '39) on the production and prevention of fatty livers have utilized rats 100 to 200 gm. in weight in which hemorrhagic degeneration has not been reported. Furthermore, in such experiments it has been customary to use high fat (40%) and very low protein (5%)

[3] More recent observations indicate that these kidneys may have been hemorrhagic very early in the experimental period and were actually in the "recovered" stage.

rations of a type unsuitable for young rats. The results obtained by these two experimental procedures have been characterized for the most part by their uniformity rather than by differences although there is as yet no certainty that the many factors which control the relation of choline to hemorrhagic degeneration in young rats are the same as those which affect liver fat in older animals. In this connection it is of interest that Tucker, Treadwell and Eckstein ('40) have recently reported that the lipotropic action of a diet containing 20% casein could be duplicated by the proper supplements of methionine and cystine in a diet containing 5% casein. Also Channon, Manifold and Platt ('40) have found that the daily intake of more than 4 mg. of cystine exerted no additional effect in promoting the deposition of liver fat in older rats.

In the present study supplementary methionine or protein methionine prevented hemorrhagic degeneration and, therefore, satisfactorily replaced choline in the diet of young rats. This result confirmed the suggestion of Griffith and Wade ('40) that protection on high casein diets may have been due to the high methionine content of such diets and supported the conclusion of du Vigneaud, Chandler, Moyer and Keppel ('39) that methionine may be a precursor of choline and may serve as a source of required methyl groups. Under the conditions of this investigation, diets containing 0.8% of methionine supplied sufficient amounts of this amino-acid for the synthesis of choline and for other indispensable functions. In the absence of added choline, hemorrhagic degeneration occurred if the diet contained less than 0.8% of methionine. It was not possible to state exactly the daily choline requirement for young rats except for specified food mixtures since the amount depended upon dietary methionine and other components of the ration, as well as upon the age and sex of the experimental animals (Griffith, '40 b). The minimum requirement of methionine which in these experiments was 0.8% of the diet and which Rose ('37) found to be 0.6% of the diet (for normal growth of young rats) should vary with the

ration and should depend in part upon the level of dietary choline.

In view of the probability that methionine may replace choline it might seem inadvisable to consider the latter a dietary essential. However, choline should be included in the list of indispensable nutrients for the young rat because the high level of methionine required in the absence of choline may be obtained only by the use of unnecessarily high protein levels or by the addition to the diet of methionine itself. Unpublished data indicate that such supplementary methionine, while effective, is less efficient than the theoretical equivalent of choline (on the basis of methyl groups). Furthermore, the fact that a diet containing as much as 25% of casein, one of the most protective high-methionine and low cystine proteins, required supplementary choline emphasized the necessity of additions of this substance to rations fed young rats in nutritional investigations.

The role of cystine in intensifying hemorrhagic degeneration remains unexplained. If cystine may be formed from methionine, then supplementary cystine should spare methionine so that more of the latter might be available for synthesis of choline, i.e., less choline should be needed in the diet. Actually, the reverse was true, more choline being required. This experimental result would be explicable if the metabolism of cystine directly involved that of choline. Or, if methionine were required for the metabolism of cystine, then supplementary cystine in low choline-low methionine diets should use up methionine so that less of the latter would be available for choline formation. In this connection the observation that the effect of cystine in increasing the requirement of choline was not proportional to the level of added cystine may be of considerable importance. Since 1.0% of added cystine required no more choline than 0.3% for prevention of hemorrhagic degeneration, then 0.7% of added cystine appeared to be used in such a way that choline was not involved, whereas 0.3% (or some smaller amount) required the simultaneous metabolism of choline. The rate of absorption from the

gastrointestinal tract was probably not a limiting factor in these experiments in which supplementary cystine (15 to 50 mg. per day) was consumed with the food inasmuch as the rate of absorption of this amino acid in rats has been found to be approximately 30 mg. per hour per 100 gm. of body weight (Wilson, '30).

Womack, Kemmerer and Rose ('37) demonstrated that cystine was dispensable in a ration which was an adequate source of methionine. However, Rose ('37) noted that a supplement of cystine greatly improved the quality of the diet for growth if methionine was supplied in insufficient amounts. Such a conception of the value of dietary cystine is involved in the following tentative explanation of the injurious effect of cystine which was observed on diets low in choline, in methionine and in cystine. On such diets there was definitely too little methionine to satisfy the demand for choline and possibly also for cystine so that the metabolism of the rat may have been limited by a double deficiency. A supplement of cystine, instead of sparing methionine, might have increased the need for methionine and choline by improving the state of nutrition. In other words the alleviation of the cystine deficiency might have aggravated the choline deficiency because of increased metabolism or because of a closer approach to normal metabolism. It has been apparent from the start of this investigation that signs of choline deficiency were most acute on low choline, low methionine diets which were superior in other respects (Griffith and Wade, '39). The proposed role of cystine obviates the necessity of assuming a direct connection between the metabolism of cystine on the one hand and that of methionine and choline on the other and also obviates the necessity of postulating a direct relation between choline and a part, but not all, of the cystine. This hypothesis, which is being investigated further, would explain the similarity in results following the addition to diets of 0.3 and 1.0% cystine since the effective level would depend only upon the need for cystine.

The importance of metabolites which may serve as sources of labile and essential methyl groups is indicated in numerous current investigations dealing with apparently divergent phases of metabolism. In this respect methionine is of unusual significance in the body not only as an amino acid but also as an intermediary which appears to be related to choline, to the turnover of phospholipids (Perlman, Stillman and Chaikoff, '40), to betaines (Chandler and du Vigneaud, '40) and to creatine (du Vigneaud, Chandler, Cohn and Brown, '40; Borsook and Dubnoff, '40). In addition methionine may play a role in the detoxication of selenium (Lewis, Schultz and Gortner, '40) and of other toxic compounds (Stevenson and White, '40). Hydrogen sulphide formed by micro-organisms in the intestinal tract (Wohlgemuth, '05) may also require methylation. It is of interest that dimethyl sulphone has been identified as a tissue constituent (Ruzicka, Goldberg and Meister, '40; Pfiffner and North, '40). The prevention of hemorrhagic degeneration in young rats is a relatively sensitive test for those metabolites which may supply methyl groups for the synthesis of choline. This aspect of the general problem of biological methylations and demethylations is being continued.

SUMMARY

1. Hemorrhagic degeneration in young rats is absent or slight if a low choline diet contains more than 0.8% of methionine, regardless of whether this level is attained by supplementary methionine or by protein levels high in casein, lactalbumin or fibrin. These results support the conclusion that methionine supplies methyl groups for the synthesis of choline.

2. Choline is a dietary essential if the ration contains less than 0.8% of methionine.

3. The choline requirement varies inversely with the dietary methionine and is increased by dietary cystine.

4. The effect of cystine is observed only on diets low in cystine and low in either choline or methionine and is not proportional to the level of supplementary cystine. A possible explanation of the effect of cystine is proposed.

5. An exceptional increase in kidney weight results on diets containing 18% of casein and 22% of gelatin even though hemorrhagic degeneration is prevented by methionine or by choline.

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THE EFFECT OF VARIOUS AMOUNTS OF CAFFEINE ON THE GASEOUS EXCHANGE AND THE RESPIRATORY QUOTIENT IN MAN

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TWO FIGURES

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Numerous investigations have been made on the effect of caffeine on various physiological processes but only a few have been concerned with the effect of the drug on the respiratory exchange including the respiratory quotient in man. Studies in which caffeine was administered in the form of coffee or tea may be excluded from consideration because of the extraneous factors that were necessarily involved. Edsall and Means ('14), Higgins and Means ('15), and Means, Aub and DuBois ('17) found that caffeine in relatively large doses stimulates metabolism but has no effect on the respiratory quotient. In each of these investigations the conclusions reached were based on a very small number of experiments. In the first there was only one experiment on each of two subjects in which, respectively, 0.390 and 0.324 gm. caffeine salicylate was injected subcutaneously; in the second, the data on the respiratory exchange are presented for only one experiment on each of two subjects in which caffeine sodium benzoate or caffeine sodium salicylate was injected in the amounts of 0.32, 0.40 and 0.50 gm.; in the third there was a total of five experiments on four subjects in which approximately 8.6 mg. per kilo body weight was administered by mouth in 300 cc. water. In view of the small number of experiments so far reported it was deemed advisable to reinvestigate the problem.

METHOD

The subjects of these experiments were two male adults, one of whom (W.W.) weighed 60 and the other (C.E.) 66 kg. Both used coffee regularly and in moderation but abstained from all beverages containing caffeine during the 24 hours preceding an experiment. The subject came to the laboratory in the fasting state at about 7 : 30 in the morning, having taken no food after 7 o'clock the preceding evening. He then reclined for 30 to 45 minutes before the experiment was begun.

The respiratory exchange was determined by the open circuit method of Carpenter and Fox ('31) with a slight modification as described elsewhere (Bachmann and Haldi, '37). Aliquot samples of expired air collected over consecutive 15-minute periods were analyzed for oxygen and carbon dioxide with the Haldane-Henderson analyzer. Check determinations were made on each sample by two analysts.

After the preliminary rest the gaseous exchange was determined for three basal periods. The caffeine dissolved in 200 cc. water at 37°C., or the same amount of water alone in the control experiments, was then ingested. The determination of gaseous exchange was resumed immediately and continued for seven periods (1¾ hours). The recumbent position was maintained throughout the experiment except for a period of 3 or 4 minutes when the subject sat up to ingest the water or solution. Three different dosages of caffeine were used, namely: 1, 3 and 6 mg. per kilo body weight. Five experiments with each dosage and the same number of controls were done on each subject, or a total of forty experiments. Care was taken that the experiments with caffeine were so spaced that there would be no after-effect of the preceding experiment.

RESULTS

The rise of the respiratory quotient above the average basal for the seven 15-minute post-ingestion periods, together with the increase in the oxygen consumption and carbon dioxide elimination are shown in graphic form in figure 1. Each point

on the curves represents an average obtained from five experiments.

Respiratory quotient. Six milligrams caffeine per kilo produced a distinct rise in the respiratory quotient. In one subject the rise reached its peak the first 15-minute period and in

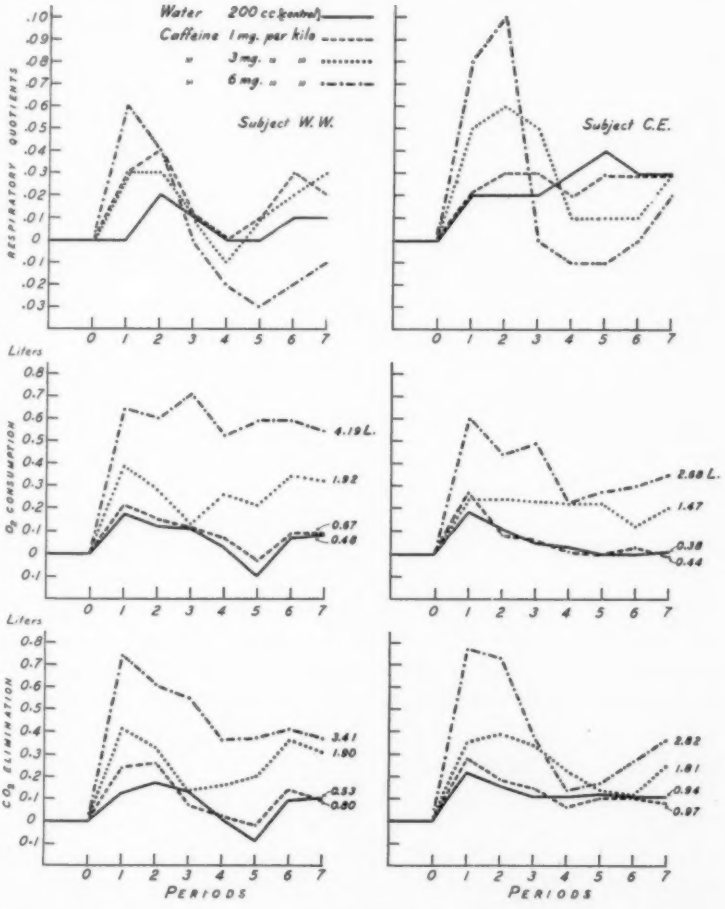

Fig. 1 The course of respiratory metabolism with reference to basal conditions and control experiments, following the administration of various doses of caffeine.

the other the second period after ingestion. Immediately after attaining its highest level the quotient fell and in the fifth period was well below the base line. In the seventh period at the conclusion of the experiment it had returned practically to the basal level.

The 3 mg. per kilo dose also was followed by an elevation of the respiratory quotient which, however, was not as pronounced as with the larger dose. The quotient followed the same general course as when 6 mg. caffeine per kilogram was taken. It is of interest to note that with the lesser rise in the quotient following the smaller dosage there was subsequently a lesser dip below the base line.

One milligram caffeine per kilo had no effect on the quotient of C.E. whereas it produced a rise practically the same as that produced by 3 mg. per kilo in the quotient of W.W. Apparently the two subjects had a different threshold of sensitivity to the drug.

Oxygen consumption. Metabolism was definitely stimulated by the two larger doses of caffeine as shown by the rise in the oxygen consumption. The stimulating effects were still evident at the end of the seventh period when the experiment was concluded. The figures on the right-hand side of the graphs give the number of liters of oxygen consumed in excess of basal requirements, on the assumption that the basal level determined for three periods preceding ingestion remained the same throughout the experiment. The average increase in the oxygen consumption by the two subjects after 6 mg. per kilo was almost exactly twice the increase after 3 mg. per kilo. It is evident from inspection of the graphs that 1 mg. caffeine per kilo had no effect on the oxygen consumption as the total increase over the control experiments was only 150 cc. for one subject and 60 cc. for the other throughout the period of 1¾ hours.

The total heat production of W.W. for the entire experiment after taking 3 mg. and 6 mg. per kilogram body weight was, respectively, 4.9 and 16.7% higher than in the control experiments and that of C.E. 5.5 and 11.4% higher. These figures,

however, do not represent the total increase in heat production resulting from the ingestion of caffeine, for the rate of oxygen consumption at the conclusion of the experiment was still above that of the control experiments. Our results are of the same order as those of Means, Aub and DuBois ('17) who found an increase of 7 to 23% in the heat production of four normal subjects over a 2-hour period after taking 8.6 mg. caffeine per kilogram body weight. The smallest dosage of 1 mg. per kilogram in our experiments had no effect on heat production; the average difference in these and the control experiments was less than 1%.

Carbon dioxide elimination. The carbon dioxide in the expired air increased after taking caffeine in proportion to the amount ingested. One milligram per kilogram had no effect on the carbon dioxide elimination of C.E. and only a small effect on that of W.W. In the latter subject 270 cc. more carbon dioxide was eliminated after taking 1 mg. caffeine per kilogram than in the control experiments. Practically all the excess elimination occurred in the first and second post-ingestion periods. While it was relatively small it was sufficient to account for the rise in the respiratory quotient in the early part of the experiment as shown in the graphs. An interesting difference is observed in the carbon dioxide elimination and oxygen consumption curves with the largest dosage of caffeine. After reaching a peak, the former drop abruptly whereas the latter decline gradually.

Interpretation of the respiratory quotient. In order to interpret the rise in the respiratory quotient induced by caffeine, it is necessary to consider the various physiological processes capable of raising the quotient and to determine which might have been affected by caffeine. The various possibilities considered were: An increase in production and accumulation of lactic acid which would liberate carbon dioxide; stimulation of the respiratory center with a consequent hyperventilation; and an increase in carbohydrate oxidation in which case the quotient would be a true combustion quotient.

In these experiments there was no accumulation of blood lactic acid. In five of the experiments in which 6 mg. per kilogram was taken, blood was drawn from the antecubital vein at the conclusion of the third basal period and again at the end of the first post-ingestion period when the respiratory quotient was at or near the peak. The average basal blood lactic acid was found to be 11.3 mg. per cent and after taking

TABLE 1

Respiratory ventilation (liters per minute at 0°C. and 760 mm.) as affected by different amounts of caffeine[1]

SUBJECT W. W.

	BASAL	CONSECUTIVE 15-MINUTE PERIODS						
		1	2	3	4	5	6	7
Control expts. (200 cc. H_2O)	5.63	+ .32	+ .40	+ .24	+.03	—.15	+.13	+.32
1 mg. caffeine per kg.	5.45	+ .62	+ .66	+ .32	+.43	+.20	+.61	+.52
3 mg. caffeine per kg.	5.42	+ .82	+ .61	+ .44	+.56	+.54	+.81	+.60
6 mg. caffeine per kg.	5.69	+1.37	+1.41	+1.18	+.93	+.91	+.84	+.85

SUBJECT C. E.

Control expts. (200 cc. H_2O)	5.81	+ .42	+ .29	+ .21	+.25	+.31	+.23	+.23
1 mg. caffeine per kg.	5.84	+ .48	+ .21	+ .09	—.01	+.08	+.02	+.03
3 mg. caffeine per kg.	5.46	+ .57	+ .92	+ .44	+.31	+.34	+.34	+.41
6 mg. caffeine per kg.	5.43	+1.35	+1.47	+1.13	+.59	+.51	+.78	+.86

[1] Each value in the table is an average obtained from five experiments.

caffeine 10.8 mg. per cent, the difference between the basal and post-ingestion samples in the individual experiments varying from 0.2 to 0.9 mg. per cent.

The generally accepted belief that caffeine acts on the respiratory center would suggest that the drug in sufficient amounts might induce hyperventilation with excessive loss of carbon dioxide. It is sometimes stated, however, that caffeine

increases the rate but not the depth of respiration (Cushny, '36). Under these circumstances it is conceivable that the stimulating action of caffeine on respiration might not induce hyperventilation. In interpreting the respiratory exchange in these experiments it is therefore necessary to take into account the minute rate and volume of respired air. These data are presented in tables 1 and 2. Since the results are more definite

TABLE 2

Average changes in the minute respiratory rate after taking caffeine[1]

SUBJECT W. W.

	BASAL	CONSECUTIVE 15-MINUTE PERIODS						
		1	2	3	4	5	6	7
Control expts. (200 cc. H_2O)	12.9	+1.3	+0.9	+1.2	+0.5	0.0	+0.2	+0.6
1 mg. caffeine per kg.	12.9	+0.3	+1.0	+0.6	+0.7	+0.5	+1.3	+0.5
3 mg. caffeine per kg.	12.6	+1.3	+1.1	+0.7	+0.9	+1.1	+1.6	+0.9
6 mg. caffeine per kg.	12.8	+1.3	+1.2	+0.8	+1.0	+1.8	+1.7	+1.6
SUBJECT C. E.								
Control expts. (200 cc. H_2O)	12.8	+0.2	+0.5	0.0	−0.8	−0.4	−0.4	−0.5
1 mg. caffeine per kg.	12.5	+0.3	−0.4	−0.2	−0.2	−0.2	−0.4	−0.6
3 mg. caffeine per kg.	12.3	+0.8	+0.9	+0.5	+0.9	+0.5	−0.2	+0.6
6 mg. caffeine per kg.	12.2	+0.4	+1.3	+1.3	+1.2	+0.9	+1.3	+1.6

[1] The minute respiratory rate was obtained by dividing by 15 the number of respirations recorded on a kymograph over 15-minute periods.

and unequivocal with the experiments in which 6 mg. of caffeine per kilogram was taken, these experiments will alone be considered in this interpretation. In these experiments the minute volume of expired air during the first three post-ingestion periods showed a much more pronounced increase than in the corresponding periods of the control experiments (table 1). The respiratory rate in these periods, however, was

not appreciably different from the rate in the controls (table 2). The depth of respirations and the tidal volume were therefore markedly increased. In the first and second post-ingestion periods the average increase in the tidal volume of the two subjects was 75 and 65 cc., respectively, whereas in the control experiments it was only 4 and 3 cc. In the last two periods of the experiments with the largest dose of caffeine the tidal volume had practically returned to normal; for the two subjects it was 10 cc. and 9 cc., respectively, above the basal as compared with 18 cc. and 15 cc. in the control experiments. Although the tidal volume was practically the same in the last two periods as in the control experiments, the minute volume of respired air was somewhat higher. This can be accounted for by the higher rate of respiration in the caffeine experiments during these periods. The larger minute volume as compared with the control experiments was most likely due to the increase in oxygen consumption which was still in evidence as shown in figure 1 at the conclusion of the experiment.

These observations on the respiratory exchange indicate that caffeine stimulated the respiratory center and thereby produced hyperventilation with excessive elimination of carbon dioxide. It may be concluded therefore that the rise in the respiratory quotient was due in part if not altogether to hyperventilation. Confirmatory evidence was obtained from determinations of the alveolar carbon dioxide. Three additional experiments were run on each subject in which alveolar air samples were obtained at the end of each 15-minute period by the method described by Carpenter and Lee ('33). In these experiments, which are not included in figure 1, the respiratory quotients followed the same general course as those in the graphs. The average basal alveolar carbon dioxide of W.W. was 5.93%. At the end of the first and second periods following the ingestion of 6 mg. caffeine per kilogram it had fallen to 5.40 and 5.51% and at the end of the fourth period had risen to 5.62%. Similar results were obtained with the other subject. The alveolar carbon dioxide percentage which was 6.42 in the basal periods fell to 6.12 and 5.99 in the first

and second periods after taking caffeine and rose to 6.23 in the fifth period.

The abrupt descent of the respiratory quotient in the third period which carried the quotient in the 6 mg. per kilogram experiments well below the base line, may be interpreted as indicating a compensatory retention of carbon dioxide after the hyperventilation which was responsible for the earlier rise in the quotient. It might be objected that this conclusion is not borne out by the course of the carbon dioxide elimination curves since they do not descend below those of the control experiments in the same manner as the respiratory quotient

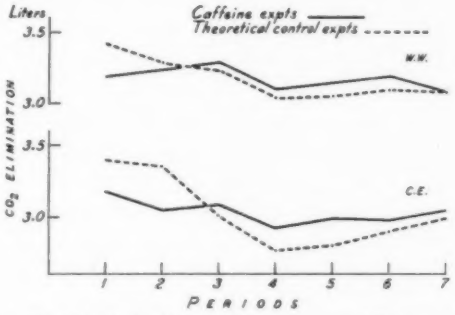

Fig. 2 Carbon dioxide elimination in the 6 mg. per kilogram experiments and in theoretical control experiments in which the same increase in oxidation as in the caffeine experiments has been assumed (see text).

curves. Further consideration, however, shows that this is not a valid objection. It is extremely important to take into account the fact that caffeine induced an increase in oxygen consumption. The curves of carbon dioxide elimination, therefore, should not be compared with the curves actually obtained in the control experiments but with theoretical curves that would have been obtained if metabolism, as measured by oxygen consumption, had been stimulated to the same extent as in the caffeine experiments, the respiratory quotients remaining the same as those obtained in the control experiments. Such theoretical curves have been constructed and are presented as solid lines in figure 2, together with curves plotted

from data in the 6 mg. per kilogram experiments. The latter curves are plotted as broken lines. It will be observed that the relationship between the curves is the same as that which obtained between the curves of the respiratory quotient in the 6 mg. caffeine per kilogram and control experiments (compare fig. 1). The carbon dioxide elimination confirms therefore the conclusion drawn from the respiratory quotient, namely, that caffeine produced a stimulation of the respiratory center which resulted in a blowing off of carbon dioxide. Later in the experiment there was a compensatory retention of carbon dioxide which was complete at the end of the experiment. An algebraic summation of the differences between the values represented by the two curves for the seven periods revealed a total difference of only 30 cc. for one subject and 40 cc. for the other.

There remains to take into account the possibility of an increase in carbohydrate oxidation concomitant with hyperventilation. When the total carbon dioxide elimination for the seven post-ingestion periods was divided by the total oxygen consumption in the various experiments, it was found that the post-ingestion respiratory quotient of W.W. in the control experiments was 0.01 above the basal and 0.01, 0.01 and 0.00 higher, respectively, in the 1, 3 and 6 mg. caffeine per kilogram experiments; the post-ingestion quotient of C. E. was 0.03 higher than the basal in the controls and 0.02, 0.03 and 0.02 higher, respectively, in the caffeine experiments. The rise in the respiratory quotients after taking caffeine consequently did not represent a stimulation of carbohydrate metabolism but was due entirely to hyperventilation. The proportion of the foodstuffs metabolized was not affected by the stimulating action of caffeine.

DISCUSSION

The results of these experiments differ from those reported by other workers in that caffeine in relatively large doses produced in our subjects a definite rise in the respiratory quotient. The difference in the results as compared with those of Means,

Aub and DuBois ('17) is probably due to the circumstance that our quotients were determined for 15-minute periods whereas theirs were calculated for periods of 1 hour. The rise of the quotients in our experiments, which is shown in figure 1, occurred in the first two periods and was followed in the next two periods by a descent below the basal level and also below the quotient in the corresponding periods of the control experiments. The rise was therefore largely counter-balanced by the subsequent drop. If the respiratory quotients had been calculated from the gaseous exchange of the first hour after taking caffeine, the increase above the basal for the two subjects in the experiments with 6 mg. per kilogram would have been only 0.02 and 0.04, respectively, as compared with a maximum of 0.06 and 0.10 in the first and second 15-minute periods.

In the other studies that report no rise in the respiratory quotient after taking caffeine, only one or two experiments were done on each subject and these experiments had no controls in which water alone was taken. It is our experience that it is hazardous to draw conclusions from so few experiments considering the unavoidable variations that are known to occur under the most carefully controlled experimental conditions. In confirmation of the work of others (Carpenter and Fox, '30) we have occasionally observed variations in the R.Q. of several points in either direction after taking 150 or 200 cc. of water at body temperature. These variations may be due to slight movements or to changes in muscle tone that cannot be readily detected (Carpenter, Hoskins and Hitchcock, '34).

Another factor that may influence the respiratory exchange is dozing on the part of the subject, for it has been shown that during sleep the oxygen consumption may decrease more than 15% (Mason and Benedict, '34). We have found that the respiratory quotient may also be affected. This may be accounted for by shallowness of respirations which may lead to hypoventilation. Training of the subject and watchfulness on

the part of the experimenter by using some simple device to make sure the subject stays awake are not always successful in preventing momentary sleep at some phase of a long experiment.

It follows from these considerations that complete reliance cannot be placed on the data of respiratory exchange unless averages are drawn from a sufficient number of carefully controlled experiments. If in spite of all precautions, an experiment is included in which vitiating factors were present but could not be definitely ascertained, the error thus introduced is greatly reduced and may become insignificant when an average is drawn from several experiments.

SUMMARY AND CONCLUSIONS

The effect of caffeine on the respiratory exchange has been studied on two subjects with dosages of 1, 3 and 6 mg. caffeine alkaloid per kilogram body weight.

One milligram caffeine per kilogram produced no effect on the oxygen consumption, carbon dioxide elimination or respiratory rate on either subject, nor in the respiratory quotient or total ventilation in one subject. In the other subject it produced a rise in the respiratory quotient and a slight increase in pulmonary ventilation.

The two larger doses were followed in the first and second 15-minute periods by a rise in the respiratory quotient, oxygen consumption and carbon dioxide elimination in both subjects. The rise in the quotient was followed by an abrupt descent which carried the quotient below the base line and below the level of the corresponding periods in the control experiments. The effects were more pronounced with the 6 mg. than with the 3 mg. per kilogram dose.

There was but little if any change in the respiratory rate following the ingestion of caffeine. In one subject there appeared to be a slight increase after the 6 mg. per kilogram dose. The tidal volume, however, showed a definite increase

with the 3 mg. and a pronounced increase with the 6 mg. per kilogram dose. The depth of respiration was therefore increased by the larger doses of caffeine, evidently the result of a stimulation of the respiratory center.

It is concluded that the rise in the respiratory quotient was due solely to the blowing off of preformed carbon dioxide.

Hyperventilation in the first two 15-minute post-ingestion periods was followed by a compensatory retention of carbon dioxide which was completed at the conclusion of the experiment.

The increase in metabolism produced by the larger doses of caffeine was not accompanied by a change in the proportion of the foodstuffs oxidized.

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